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March 30, 2001

## BOX PCT

Commissioner for Patents  
Washington, D.C. 20231PCT/JP99/05346  
-filed September 29, 1999

Re: Application of Shigeru YAMAMOTO, Masamichi OKADA, Taichi USUI, Kanzo SAKATA, Atsuki TOUMOTO and Kazutaka TSURUHAMI  
NOVEL ENZYME COMPOSITION AND PRODUCTION METHOD AND USE THEREOF  
Our Ref: Q63731

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☒ an executed Declaration and Power of Attorney.
- ☒ an English translation of the International Application.
- ☒ Notification Concerning Submission or Transmittal of priority document.
- ☒ Statement to Support Filing and Submission in accordance with 37 C.F.R. §§ 1.821-1.825
- ☒ an executed Assignment and PTO 1595 form.
- ☒ International Search Report, PTO Form 1449 listing the ISR references, and a complete copy of each reference.
- ☒ a Preliminary Amendment

It is assumed that copies of the International Application, the International Search Report, the International Preliminary Examination Report, and any Articles 19 and 34 amendments as required by § 371(c) will be supplied directly by the International Bureau, but if further copies are needed, the undersigned can easily provide them upon request.

The Government filing fee is calculated as follows:

Total claims	30	-	20	=	10	x	\$18.00	=	\$180.00
Independent claims	9	-	3	=	6	x	\$80.00	=	\$480.00
Base Fee									\$860.00
Multiple Dependent Claim Fee									\$270.00

<b>TOTAL FILING FEE</b>	\$1790.00
<b>Recordation of Assignment</b>	\$ 40.00
<b>TOTAL FEE</b>	\$1830.00

Checks for the statutory filing fee of \$1790.00 and Assignment recordation fee of \$40.00 are attached. You are also directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880. The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Priority is claimed from September 30, 1998 based on JP Application No. P. Hei. 10-294675.

Respectfully submitted,

*Paul E. Bolland*  
Mark Bolland  
Registration No. 32,197

Q63731/plr

## PATENT APPLICATION

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Shigeru YAMAMOTO, et al.

Appln. No.: Not Yet Assigned

Group Art Unit:

Confirmation No.: Not Yet Assigned

Examiner:

Filed: March 30, 2001

For: NOVEL ENZYME COMPOSITION AND PRODUCTION METHOD AND USE  
THEREOF

## PRELIMINARY AMENDMENT

Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-identified application as follows:

**IN THE SPECIFICATION:**

**Page 21-22 please delete the third full paragraph and insert the following new paragraph:**

The hybridization can be carried out under the aforementioned stringent condition. For example, DNAs from a genomic DNA library or a cDNA library obtained from a microorganism capable of producing an enzyme of the present invention is fixed on nylon membranes, and the thus prepared nylon membranes are subjected to blocking at 65°C in a pre-hybridization solution containing 6 x SSC, 0.5% SDS, 5 x Denhart's and 100 µg/ml of salmon sperm DNA. Thereafter, each probe labeled with <sup>32</sup>P or digoxigenin is added thereto, followed by incubation overnight at 68°C. The thus treated nylon membranes are washed in 6 x SSC containing 0.1% SDS at room temperature for 10 minutes, in 6 x SSC containing 0.1% SDS 45°C for 30 minutes and then

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subsequently subjecting the thus washed membranes to an auto-radiography or detection of digoxigenin to detect a DNA fragment which hybridizes with the probe in a specific fashion. Also, genes which show various degree of homology can be obtained by changing certain conditions such as washing or lowering the hybridization temperature (e.g., 45°C).

**Page 22 please delete the second full paragraph and insert the following new paragraph:**

In order to determine whether the thus obtained gene encodes a polypeptide having the enzyme activity of interest, the thus determined nucleotide sequence is compared with the nucleotide sequence coding for the enzyme of the present invention or with its amino acid sequence, and the identity is estimated based on the gene structure and homology. Alternatively, it is possible to determine whether the gene encodes a polypeptide which has the enzyme activity of interest by producing a polypeptide encoded by the gene and measuring its enzyme activity.

**Page 32, please delete the second full paragraph and insert the following new paragraph:**

Example 2

In accordance with Example 1, *Aspergillus fumigatus* IAM 2046 was pre-cultured in the same manner, and the resulting culture broth was inoculated into a main culture medium (2% soybean flour, 0.3% sodium chloride, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 3% soluble starch, 0.5% gentose #80 (mfd. by Nihon Shokuhin Kako), pH 5.6) and cultured for 4 days to obtain a crude enzyme solution. As the result, the

**Page 33, please delete table 3 and insert the following new table:**

Table 3

Inducers	Inducing ability (%)
Not added	100
Isomaltose	145
Maltotriose	171
Maltose	136
Gentose #80	235
Gentiobiose	211
Gentio-oligosaccharide	180
Sucrose	116
Trehalose	113
Glucose	164
Galactose	125
Fructose	143
Rhamnose	129
Tulbose	116
Maltitol	142
Arabitol	112
Galactitol	142
Glucosamine hydrochloride	157

**Page 48, please delete the first full paragraph and insert the following new paragraph:**

Its optimum temperature was measured in the following manner. A 400 µl portion of 2 mM pNP-primeveroside solution prepared using 20 mM disodium citric acid-HCl buffer (pH 2.5) was mixed with 90 µl of the enzyme solution to carry out the reaction at 30 to 65°C for 20 minutes. The reaction was stopped by adding 500 µl of 0.5 M sodium carbonate solution, and the activity was determined by measuring the absorbance at 420 nm. It was found that sufficient activity is maintained, because diglycosidase derived from *Aspergillus fumigatus* has 80% of the activity even at 60°C, in comparison with the plant-derived enzymes having similar activity.

**Page 48, please delete the second full paragraph and insert the following new paragraph:**

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Attorney Docket No. Q63731

Its pH stability was measured in the following manner. The purified enzyme preparation was diluted 100 times with each of disodium citrate buffer of from pH 2 to 5, phosphate buffer of from pH 6 to 8 or glycine NaCl-NaOH buffer of from pH 7 to 10 and treated at 37°C for 1 hour, and then a 90 µl portion thereof was mixed with 400 µl of 2 mM pNP-primeveroside solution (pH 2.5) which had been incubated at 37°C for 5 minutes, and the reaction was carried out at 37°C for 20 minutes. The reaction was stopped by adding 500 µl of 0.5 M sodium carbonate

**Page 63, please delete the first full paragraph and insert the following new paragraph:**

In order to verify whether or not the cloned gene is the primeverosidase gene, expression of the thus obtained DNA was confirmed. Using an *Aspergillus oryzae* Taka-amylase gene-containing plasmid pTG-Taa (Kato M, Aoyama A, Naruse F, Kobayashi T and Tsukagoshi N (1997), An *Aspergillus nidulans* nuclear protein, An CP, involved in enhancement of Taka-amylase A gene expression binds to the CCAAT-containing *taaG2*, *amdS* and *gatA* promoters., *Mol. Gen. Genet.*, 254: 119 - 126) as the template, a fragment was obtained by amplifying it by PCR using a primer TAA5'

SEQ ID NO: 11

sense primer:

5'-GGGCCTGCAGGAATTCATGGTGT-3'

and a primer TP3'

SEQ ID NO: 12

antisense primer:

5'-CGAGCCGGGGTTCCGTCCGAGGCGTTC-3'.

<PCR reaction solution>

template DNA solution (50 µg/ml)

1 µl

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50  $\mu$ M sense primer 1  $\mu$ l

50  $\mu$ M antisense primer 1  $\mu$ l

**Page 64, please delete the first full paragraph and insert the following new paragraph:**

Also, a fragment was obtained by amplifying it by PCR using the DNA-containing plasmid pAFPri as the template and using a primer dPC5'

SEQ ID NO: 13

sense primer:

5'-GCAACGCCTGCGGACGGAAACCCCGGCTCG-3'

and a primer PC3'

SEQ ID NO: 14

antisense primer:

5'-GCGCAAGCTTGAAGCTGCACGACATGTAA-3'.

**Page 65, please delete the first full paragraph and insert the following new paragraph:**

Regarding the terminator region, a fragment was obtained by amplifying it by PCR using pTG-Taa as the template and using a primer TAAH

SEQ ID NO: 15

sense primer:

5'-GCGCAAGCTTTGAAGGGTGGAGAGT-3'

and a primer TAA3'

SEQ ID NO: 16

antisense primer:

5'-GCGCCCTGCAGGTCTAGAATTCCTAGTGGTT-3'.

**Page 66-67, please delete the second paragraph and insert the following new paragraph:**

An orotidine-5'-phosphate decarboxylase (PyrG ) producing strain *Aspergillus nidulans* G191 (Kato M (1997), *Mol. Gen. Genet.*, 254: 119 - 126) was inoculated into a complete medium (2% malt extract, 0.1% peptone, 2% glucose, 0.1% uridine, 2 µg/ml p-aminobenzoic acid, pH 6.5) and cultured at 30°C for 18 hours on a shaker. The cells were collected by filtration, suspended in a protoplast solution (0.8 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM CaCl<sub>2</sub>, 3.75 mg/ml Novozyme 234) and then treated at 30°C for 1 hour on a shaker. The resulting protoplasts were recovered by filtration and centrifuged at 1,500 rpm for 5 minutes to obtain the protoplasts as the precipitate. This precipitate was suspended in 0.8 M NaCl solution and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. This was again suspended in 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. A protoplast solution was obtained by suspending this in an appropriate amount of 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution. Next, 50 µl of this protoplast solution was mixed with 20 µg of a DNA solution and 12.5 µl of a PEG solution (25% PEG 6000/50 mM CaCl<sub>2</sub>/10 mM Tris-HCl (pH 7.5)) and then allowed to stand for 20 minutes on ice. Next, 0.5 ml of PEG solution was added and then the mixture was allowed to stand for 5 minutes on ice. Next, 1 ml of 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution was added and mixed. A 0.5 ml portion of this mixed solution was mixed with 15 ml of 2% agar-containing regeneration medium (0.6% NaNO<sub>3</sub>, 11 mM KH<sub>2</sub>PO<sub>4</sub>, 7 mM KCl, 1.2 M sorbitol, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1% glucose, 2 µg/ml p-aminobenzoic acid, pH 6.5) which had been incubated at 50°C in advance, solidified in Petri dishes and then cultured at 37°C for 3 days.

**Page 76, please delete the first full paragraph and insert the following new paragraph:**

Example 16

Hydrolysis of other disaccharide glycosides

Using the diglycosidase preparations derived from various microorganisms shown in Example 5, their ability to hydrolyze various disaccharide glycosides was examined using TLC. As a result, it was revealed that the diglycosidase acts upon not only the primeveroside glycosides but also various other disaccharide glycosides analogous to the primeveroside glycosides, including rutinose glycosides such as naringin and rutin, gentiobiose glycosides, arabinofuranosyl glycosides and apiofuranosyl glycosides, and thereby releases disaccharides and produces respective free aglycons.

**Page 79, please delete the second full paragraph and insert the following new paragraph:**

The purified enzyme of Example 6 was diluted with deionized water to adjust the activity to 1.0 AU/ml, and acetonitrile (200  $\mu$ l) containing 2.5% phenethyl alcohol, 20 mM acetate buffer (250  $\mu$ l) containing 10% primeverose and the enzyme solution (50  $\mu$ l) were mixed to carry out the reaction at 55°C. The reaction was completed after 6 hours and the reaction solution was mixed with 500  $\mu$ l of diethyl ether, stirred and then centrifuged to remove free aglycon which was transferred into the ether layer. By applying the water layer to a Diaion HP-20 column and passing purified water through the column, free primeverose was removed. The disaccharide glycoside adsorbed to the resin was eluted with methanol and concentrated to dryness. This was dissolved in 100  $\mu$ l of deionized water, and a 20  $\mu$ l portion thereof was spotted on a TLC plate to detect the reaction product (the developing solvent was ethyl acetate:acetic acid:deionized water = 3:1:1, acetic

#### **REMARKS**

The amended paragraphs submitted herewith correct misspellings and obvious errors. In addition, the amended paragraphs correct the numbering of the four sequencing primers on pages 63-65. The numbering of sequences is correct in the Sequence Listing filed concurrently herewith.



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Attorney Docket No. Q63731

Therefore, no new matter has been added. Entry and consideration of this Amendment is respectfully requested.

Respectfully submitted,



Drew Hissong  
Registration No. 44,765

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Date: March 30, 2001

09806413-033001

**APPENDIX**

**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**IN THE SPECIFICATION:**

**The specification is changed as follows:**

**Page 21-22 Third full paragraph:**

The hybridization can be carried out under the aforementioned stringent condition. For example, DNAs from a genomic DNA library or a cDNA library obtained from a microorganism capable of producing an enzyme of the present invention is fixed on nylon membranes, and the thus prepared nylon membranes are subjected to blocking at 65°C in a pre-hybridization solution containing 6 x SSC, 0.5% SDS, 5 x Denhart's and 100 µg/ml of salmon sperm DNA. Thereafter, each probe labeled with <sup>32</sup>P or digoxigenin is added thereto, followed by incubation overnight at 68°C. The thus treated nylon membranes are washed in 6 x SSC containing 0.1% SDS at room temperature for 10 minutes, in 6 x SSC containing 0.1% SDS 45°C for 30 minutes and then subsequently subjecting the thus washed membranes to an auto-radiography or detection of digoxigenin to detect a DNA fragment which hybridizes with the probe in a specific fashion. Also, genes which show various degree of homology can be obtained by changing certain conditions such as washing or lowering the hybridization temperature (e.g., 45°C).

**Page 22 2<sup>nd</sup> full paragraph:**

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it is possible to determine whether the gene encodes a polypeptide which has the enzyme activity of interest by producing a polypeptide ~~of~~ encoded by the gene and measuring its enzyme activity.

**Page 32, Second full paragraph:**

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Sucrose	116
Trehalose	113
Glucose	164
Galactose	125
Fructose	143
Rhamnose	129
Tulbose	116
Maltitol	142
Arabitol	112
Galactitol	142
Glucosamine hydrochloride	157

**Page 48, First full paragraph:**

Its optimum temperature was measured in the following manner. A 400 µl portion of 2 mM pNP-primeveroside solution prepared using 20 mM secondary disodium citric acid-HCl buffer (pH 2.5) was mixed with 90 µl of the enzyme solution to carry out the reaction at 30 to 65°C for 20 minutes. The reaction was stopped by adding 500 µl of 0.5 M sodium carbonate solution, and the activity was determined by measuring the absorbance at 420 nm. It was found that sufficient activity is maintained, because diglycosidase derived from *Aspergillus fumigatus* has 80% of the activity even at 60°C, in comparison with the plant-derived enzymes having similar activity.

**Page 48, Second full paragraph:**

Its pH stability was measured in the following manner. The purified enzyme preparation was diluted 100 times with each of secondary disodium citrate buffer of from pH 2 to 5, phosphate buffer of from pH 6 to 8 or glycine NaCl-NaOH buffer of from pH 7 to 10 and treated at 37°C for 1 hour, and then a 90 µl portion thereof was mixed with 400 µl of 2 mM pNP-primeveroside solution (pH 2.5) which had been incubated at 37°C for 5 minutes, and the reaction was carried out at 37°C for 20 minutes. The reaction was stopped by adding 500 µl of 0.5 M sodium carbonate

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(1997), An *Aspergillus nidulans* nuclear protein, An CP, involved in enhancement of Taka-amylase A gene expression binds to the CCAAT-containing *taaG2*, *amdS* and *gatA* promoters., *Mol. Gen. Genet.*, 254: 119 - 126) as the template, a fragment was obtained by amplifying it by PCR using

a primer TAA5'

SEQ ID NO: ~~40~~ 11

sense primer:

5'-GGGCCTGCAGGAATTCATGGTGTT-3'

and a primer TP3'

SEQ ID NO: ~~44~~ 12

antisense primer:

5'-CGAGCCGGGGTTTCCGTCCGCAGGCGTTGC-3'.

<PCR reaction solution>

template DNA solution (50 µg/ml)	1 µl
50 µM sense primer	1 µl
50 µM antisense primer	1 µl

**Page 64, First full paragraph:**

Also, a fragment was obtained by amplifying it by PCR using the DNA-containing plasmid pAFPri as the template and using a primer dPC5'

SEQ ID NO: ~~42~~ 13

sense primer:

5'-GCAACGCCTGCGGACGGAAACCCCGGCTCG-3'

and a primer PC3'

SEQ ID NO: ~~43~~ 14

antisense primer:

5'-GCGCAAGCTTGAAGCTGCACGACATGTAA-3'.

**Page 65, First full paragraph:**

Regarding the terminator region, a fragment was obtained by amplifying it by PCR using pTG-Taa as the template and using a primer TAAH

SEQ ID NO: ~~44~~ 15

sense primer:

5'-GCGCAAGCTTTGAAGGGTGGAGAGT-3'

and a primer TAA3'

SEQ ID NO: ~~45~~ 16

antisense primer:

5'-GCGCCCTGCAGGTCTAGAATTCCTAGTGGTT-3'.

**Page 66-67, Second paragraph:**

An orotidine-5'-phosphate decarboxylase (~~pyrG~~) (PyrG) producing strain *Aspergillus nidulans* G191 (Kato M (1997), *Mol. Gen. Genet.*, 254: 119 - 126) was inoculated into a complete medium (2% malt extract, 0.1% peptone, 2% glucose, 0.1% uridine, 2 µg/ml p-aminobenzoic acid, pH 6.5) and cultured at 30°C for 18 hours on a shaker. The cells were collected by filtration, suspended in a protoplast solution (0.8 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM CaCl<sub>2</sub>, 3.75 mg/ml Novozyme 234) and then treated at 30°C for 1 hour on a shaker. The resulting protoplasts were recovered by filtration and centrifuged at 1,500 rpm for 5 minutes to obtain the protoplasts as the precipitate. This precipitate was suspended in 0.8 M NaCl solution

and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. This was again suspended in 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. A protoplast solution was obtained by suspending this in an appropriate amount of 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution. Next, 50 µl of this protoplast solution was mixed with 20 µg of a DNA solution and 12.5 µl of a PEG solution (25% PEG 6000/50 mM CaCl<sub>2</sub>/10 mM Tris-HCl (pH 7.5)) and then allowed to stand for 20 minutes on ice. Next, 0.5 ml of PEG solution was added and then the mixture was allowed to stand for 5 minutes on ice. Next, 1 ml of 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution was added and mixed. A 0.5 ml portion of this mixed solution was mixed with 15 ml of 2% agar-containing regeneration medium (0.6% NaNO<sub>3</sub>, 11 mM KH<sub>2</sub>PO<sub>4</sub>, 7 mM KCl, 1.2 M sorbitol, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1% glucose, 2 µg/ml p-aminobenzoic acid, pH 6.5) which had been incubated at 50°C in advance, solidified in Petri dishes and then cultured at 37°C for 3 days.

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Using the diglycosidase preparations derived from various microorganisms shown in Example 5, their ability to hydrolyze various disaccharide glycosides was examined using TLC. As a result, it was revealed that the diglycosidase acts upon not only the primeveroside glycosides but also various other disaccharide glycosides analogous to the primeveroside glycosides, including rutinose glycosides such as naringin and rutin, gentiobiose glycosides, arabinofuranosyl glycosides and ~~arabinofuranosyl~~ apiofuranosyl glycosides, and thereby releases disaccharides and produces respective free aglycons.

**Page 79, Second full paragraph:**

The purified enzyme of Example 6 was diluted with deionized water to adjust the activity to 1.0 AU/ml, and acetonitrile (200 ~~ml~~ μl) containing 2.5% phenethyl alcohol, 20 mM acetate buffer (250  $\mu$ l) containing 10% primeverose and the enzyme solution (50  $\mu$ l) were mixed to carry out the reaction at 55°C. The reaction was completed after 6 hours and the reaction solution was mixed with 500  $\mu$ l of diethyl ether, stirred and then centrifuged to remove free aglycon which was transferred into the ether layer. By applying the water layer to a Diaion HP-20 column and passing purified water through the column, free primeverose was removed. The disaccharide glycoside adsorbed to the resin was eluted with methanol and concentrated to dryness. This was dissolved in 100  $\mu$ l of deionized water, and a 20  $\mu$ l portion thereof was spotted on a TLC plate to detect the reaction product (the developing solvent was ethyl acetate:acetic acid:deionized water = 3:1:1, acetic



NOVEL ENZYME COMPOSITION AND PRODUCTION METHOD AND  
USE THEREOF

TECHNICAL FIELD

This invention relates to a novel microorganism-derived enzyme having an enzyme activity to cut disaccharide glycosides, particularly  $\beta$ -primeveroside and/or its analogs, in disaccharide unit, a method for producing said enzyme, a gene which encodes said enzyme, a vector containing said gene, a transformant transformed with said vector and use of said enzyme.

BACKGROUND ART

Alcoholic aromas such as geraniol, linalool, benzyl alcohol, 2-phenyl alcohol and  $C_{13}$ -norterpenoid alcohol as plant aroma components take an important role in the aroma formation of, for example, flowers, tea, fruits and wine.

Among these aroma components, monosaccharide glycosides such as  $\beta$ -D-glucopyranoside have been isolated and identified as aroma precursors of benzyl alcohol and (Z)-3-hexenol.

Recently, the presence of a disaccharide glycoside  $\beta$ -primeveroside (6-O- $\beta$ -D-xylopyranosyl- $\beta$ -D-glucopyranoside) or its analogs has been confirmed as precursors of fragrant alcohols such as geraniol and linalool, which seem to be



depending on the prior art supply sources such as tea leaves.

#### DISCLOSURE OF THE INVENTION

With the aim of solving the aforementioned problems, the inventors of the present invention have conducted intensive studies searching for its supply source in microorganisms. That is, as a result of efforts to screen a microorganism capable of producing an enzyme having the aforementioned function from a broad range of natural sources, we have found a microorganism which is suitable for fermentation culturing and has the ability to produce an enzyme having the action to cut the saccharide moiety of disaccharide glycosides such as  $\beta$ -primeveroside in disaccharide unit, and have isolated and purified said enzyme and determined nucleotide sequence of a gene that encodes said enzyme. Thereafter, we have found the enzyme having the aforementioned action in a large number of microorganisms such as molds, yeast, bacteria and actinomycetes and thereby accomplished this invention.

Accordingly, the invention relates to a novel microorganism-derived enzyme having an enzyme activity to cut disaccharide glycosides, particularly  $\beta$ -primeveroside and/or its analogs, in disaccharide unit, a method for producing said enzyme, a gene which encodes said enzyme, a

vector containing said gene, a transformant transformed with said vector and use of said enzyme.

The enzyme of the present invention is characterized in that it has an activity to release saccharides in a disaccharide unit from the disaccharide glycoside by acting on the disaccharide glycoside which can hardly be utilized as the substrate by the known glycosidase. In the present specification, the enzyme having such activity is called "diglycosidase". The diglycosidase of the present invention has not only the activity to act on the disaccharide glycoside to release the saccharides in a disaccharide unit but also cut the glycoside bonding of the monoglycoside. Moreover, it has an activity to cut the glycoside bonding of the modified monoglycoside (e.g., acetylglucoside, malonylglucoside, methylglucoside, phosphoglucoside, and amidoglucoside).

The microorganism-derived enzyme of the present invention which has the activity to act upon a disaccharide glycoside and thereby release saccharides in disaccharide unit from said disaccharide glycoside is different from the plant-derived enzyme in terms of physicochemical properties and homology of gene sequences.

Next, the present invention is described in detail.

In this connection, results of the measurement of various enzyme activities carried out in the present

invention are shown by values obtained by the following methods unless otherwise noted.

(1) Disaccharide glycoside degradation activity

Measurement of the activity was carried out using an automatic chemical analyzer (TBA-30R, manufactured by TOSHIBA CORP.). A 30  $\mu$ l portion of each enzyme sample was mixed with 200  $\mu$ l of acetate buffer solution (pH 5.5) containing 2 mM of p-nitrophenyl (pNP) primeveroside as the disaccharide glycoside substrate to carry out the reaction at 40°C and at a cycle time of 22.5 seconds, for 9.75 minutes, and then the reaction solution was mixed with 250  $\mu$ l of sodium carbonate to measure absorbance at 412 nm. Measurement of the sample blank was carried out in the same manner using 20 mM acetate buffer (pH 5.5) instead of the substrate solution.

One unit of the enzyme activity is defined as the amount of enzyme which increases the absorbance by a factor of 1 under these conditions.

The pNP-primeveroside used herein can be synthesized for example by allowing pNP-glucoside (manufactured by Merck) to react with xylo-oligosaccharide (manufactured by Wako Pure Chemical Industries) using an enzyme xylosidase (manufactured by Sigma), thereby effecting transfer of one xylose residue to pNP-glucoside through  $\beta$ -1,6-bonding.

(2)  $\beta$ -Glucosidase activity

Measurement of the activity was carried out using an automatic chemical analyzer (TBA-30R, manufactured by TOSHIBA CORP.). A 10  $\mu$ l portion of each enzyme sample was mixed with 200  $\mu$ l of acetate buffer solution (pH 5.5) containing 2 mM of p-nitrophenyl (pNP) glucoside as the substrate to carry out the reaction at 40°C and at a cycle time of 22.5 seconds, and then the reaction solution was mixed with 250  $\mu$ l of sodium carbonate to measure absorbance at 412 nm. Measurement of the sample blank was carried out in the same manner using 20 mM acetate buffer (pH 5.5) instead of the substrate solution.

One unit of the enzyme activity is defined as the amount of enzyme which increases the absorbance by a factor of 1 under these conditions.

In order to obtain a microorganism capable of producing an enzyme having a diglycosidase activity, the present inventors have examined a broad range of natural sources and found that several microbial strains isolated from the natural world can produce an enzyme having said activity. The disaccharide glycosides analogous to  $\beta$ -primeveroside are disaccharides having glucose on the aglycon side, such as apiofuranosyl- $\beta$ -D-glucopyranoside and arabinofuranosyl- $\beta$ -D-glucopyranoside.

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## 030643-03001

- 030643-03001

(2) Kozakiewicz, Z., 1989. *Aspergillus* species on stored products. Mycological Papers, No. 161, CAB International Mycological Institute.

(3) Al-Musallam, A., 1980. "Revision of the black *Aspergillus* species", University of Utrecht.

Mycological properties are described in the following.

Identification of strain A

(1) Growth condition

Growth condition

· Czapek agar medium

Colony size is 48 to 50 mm in diameter (25°C, 7 days), its surface is velutinous to powdery, hypha is white, formation of conidia is slightly poor, dull green to grayish green, backside is light yellowish brown to brown.

· Malt extract agar medium

Colony size is 78 to 80 mm in diameter (25°C, 7 days), its surface is velutinous powdery, hypha is white, formation of conidia is markedly good, dull green to grayish green, backside is colorless to yellowish white. Colony size at 37°C (3 days) is 73 to 75 mm in diameter. Good growth even at 45°C.



(2) Morphology

· Conidial heads:

Strong columnar form, 48 to 128  $\mu\text{m}$  in length, 16 to 52  $\mu\text{m}$  in diameter, dull green to grayish green.

· Conidiophores:

Forms from substrate mycelium, 125 to 800  $\mu\text{m}$  in length (mostly 500  $\mu\text{m}$  or less), 5 to 10  $\mu\text{m}$  in diameter, straight or slight bending, smooth surface.

· Vesicles:

Diameter from 10 to 25  $\mu\text{m}$ , flask shape, forms phialide in upper 2/3.

· Metulae:

Not formed.

· Phialides:

5.6 ~ 12 x 2.4 ~ 3.2  $\mu\text{m}$

· Conidia:

Diameter from 2.6 to 3.6  $\mu\text{m}$ , globose to subglobose, echinulate surface.

· Ascospores:

Not formed.

The above results show that the strain A belongs to the *Aspergillus fumigatus* group, because the conidium forming cells are single columnar (metula is not formed), the conidial head is cylindrical and dull green to grayish green, the conidia are globose and ascospore is not formed.

In addition, since the conidial head is strong columnar and does not form nodding appearance, the conidia have echinulate surface and most of the conidiophores are 500  $\mu$ m or less, this strain is *Aspergillus fumigatus*.

Identification of strains B, C and D

(1) Growth condition

Table 1

Medium	Item	Strain B	Strain C	Strain D
Czapek agar medium	Colony diameter (25°C, 7 days)	45 to 48 mm	47 to 50 mm	46 to 48 mm
	Colony diameter (25°C, 14 days)	80 mm or more	80 mm or more	80 mm or more
	Hyphae layer	dense, white to yellow	dense, white	dense, white
	Formation of conidia	good	good	good
	Color of conidia	dull grayish brown to black brown	dull grayish brown to black brown	dull grayish brown to black brown
	Backside color	white to yellow	white	white
Malt extract agar medium	Colony diameter (25°C)	46 to 51 mm	53 to 55 mm	55 to 59 mm
	Hyphae layer	thin and flat, colorless	thin and flat, colorless	thin and flat, colorless
	Formation of conidia	very good	very good	very good
	Color of conidia	black to black brown	black to black brown	black to black brown
	Backside color	colorless	colorless	colorless

## (2) Morphology (Czapek agar medium)

Table 2

Item		Strain B	Strain C	Strain D
Conidial heads	Shape	spherical, radial, sometimes split into cylindrical form when matured	spherical, radial, sometimes split into cylindrical form when matured	spherical, radial, sometimes split into cylindrical form when matured
	Size	120 to 560 $\mu\text{m}$	150 to 500 $\mu\text{m}$	125 to 350 $\mu\text{m}$
	Color	dull grayish brown to black brown	dull grayish brown to black brown	dull grayish brown to black brown
Conidio-phores	Origin	forms from substrate mycelium	forms from substrate mycelium	forms from substrate mycelium
	Length	350 $\mu\text{m}$ to 3 mm	350 $\mu\text{m}$ to 3 mm	350 $\mu\text{m}$ to 2.5 mm
	Diameter	9 to 20 $\mu\text{m}$	10 to 22.5 $\mu\text{m}$	12.5 to 20 $\mu\text{m}$
	Surface	smooth	smooth	smooth
Vesicles	Diameter	20 to 80 $\mu\text{m}$	15 (mostly 35) to 80 $\mu\text{m}$	30 to 80 $\mu\text{m}$
	Shape	globose	globose	globose
	Metula formation region	entire	entire	entire
Metulae	Length	20 to 24 $\mu\text{m}$	12 to 22.4 $\mu\text{m}$	12.8 to 24 $\mu\text{m}$
	Diameter	5.6 to 7.2 $\mu\text{m}$	4.8 to 6.8 $\mu\text{m}$	5.6 to 8 $\mu\text{m}$
	Shape	globose to subglobose	globose to subglobose	globose to subglobose
	Surface	echinulate	echinulate	echinulate
Ascospore		not formed	not formed	not formed

Based on the above results, all of the strains B, C and D belong to the *Aspergillus niger* group, because the conidia forming cells are double columnar (metulae and phialides are formed) and the conidial heads are globose and blackish. In addition, since the colony diameter becomes 5 cm or more by 14 days on the Czapek agar medium, the conidial surface is echinulate (verrucose), the conidium is globose to subglobose shape of 6  $\mu\text{m}$  or less and

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dull grayish brown to black brown and the conidiophore is 6  $\mu\text{m}$  or less, these are strains of *Aspergillus niger* var. *niger*.

The present inventors also have selected strains belonging to the genus *Aspergillus* from type cultures at random and examined their ability to produce diglycosidase. As a result, productivity of the enzyme was also found, for example, in *Aspergillus niger* IFO 4407, *Aspergillus niger* IAM 2020 and *Aspergillus fumigatus* IAM 2046, etc. In addition, screening of various other microorganisms was also carried out for their ability to produce diglycosidase. As a result, the diglycosidase activity was found in various microorganisms such as those belonging to the genus *Aspergillus*, the genus *Penicillium*, the genus *Rhizopus*, the genus *Rhizomucor*, the genus *Talaromyces*, the genus *Mortierella*, the genus *Cryptococcus*, the genus *Microbacterium*, the genus *Corynebacterium* and the genus *Actinoplanes*.

The strains which can be used in the present invention are not limited to the strains described above, and any strain having diglycosidase productivity can be used. In addition, mutants of the strains having diglycosidase productivity, or various microorganisms or various cells (e.g., yeast cells, bacterial cells, higher plant cells and animal cells) modified by recombinant DNA

techniques to have an ability to produce diglycosidase, in particular, preferably those modified to produce diglycosidase in great quantity are also included in the production method which can be used in the present invention. When diglycosidase productivity is added by introducing a diglycosidase gene, the host microorganism may not have the diglycosidase productivity.

When diglycosidase is produced using the aforementioned various microorganisms, methods and conditions suited for the culturing of said microorganisms can be selected, and such methods and conditions are not particularly limited. For example, culturing method of the aforementioned various strains may be either liquid culturing or solid culturing, but liquid culturing is preferably used. For example, the liquid culturing can be carried out in the following manner.

Any type of medium can be used, provided that a diglycosidase producing microorganism can be grown therein. For example, a medium to be used may contain carbon sources such as glucose, sucrose, gentiobiose, soluble starch, glycerol, dextrin, molasses and organic acids, nitrogen sources such as ammonium sulfate, ammonium carbonate, ammonium phosphate, ammonium acetate, peptone, yeast extract, corn steep liquor, casein hydrolysate, wheat bran and meat extract and inorganic salts such as potassium

salts, magnesium salts, sodium salts, phosphates, manganese salts, iron salts and zinc salts. In addition, various inducers can be added to the medium in order to produce and accumulate diglycosidase. Examples of the inducers to be used include saccharides, preferably gentose (e.g., Gentose #80, Nihon Shokuhin Kako), gentiobiose and gentio-oligosaccharide (e.g., Gentio-oligosaccharide, Wako Pure Chemicals). Amount of these inducers to be added is not particularly limited, with the proviso that the amount is effective in increasing the productivity of diglycosidase to an intended level, but is added preferably in an amount of from 0.01 to 5%.

The medium pH is adjusted to a level of approximately from 3 to 8, preferably from about 5 to 6, and the culturing is carried out under aerobic conditions at a culturing temperature of generally from about 10 to 50°C, preferably at about 30°C, for a period of from 1 to 15 days, preferably from 4 to 7 days. Regarding the culturing method, shaking culture and aerobic submerged culture by a jar fermentor can be used. However, the aforementioned various culture conditions are optionally changed depending on the microorganisms or cells to be cultured as a matter of course, and such conditions are not particularly limited with the proviso that the diglycosidase of the present invention can be produced.

Regarding the isolation and purification of diglycosidase from the thus obtained culture broth, purified primeverosidase can be obtained in the usual way by a combination of centrifugation, UF concentration, salting out and various types of chromatography such as of an ion exchange resin.

The culture of the aforementioned microorganism as it is can be used as the enzyme composition of the present invention. Of course, the culture may be purified to an appropriate degree of purification depending on the intended use of the present invention.

The following further describes a gene which encodes a microorganism-derived enzyme of the present invention having the activity to act upon a disaccharide glycoside and thereby release saccharides from said disaccharide glycoside in disaccharide unit, a recombinant vector which contains said gene, a transformant into which said vector is introduced and a method for producing said enzyme using said transformant.

As the microorganism-derived enzyme of the present invention having the activity to act upon a disaccharide glycoside and thereby release saccharides from said disaccharide glycoside in disaccharide unit, all of the enzymes which can be obtained by the aforementioned production methods are included, in which particularly

preferred one is a polypeptide which has the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing, wherein one or more amino acid residues of the amino acid sequence may be modified by at least one of deletion, addition, insertion and substitution, and more preferred one is a polypeptide which has the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing.

Examples of the gene which encodes the enzyme of the present invention include a gene which can be obtained from a microorganism capable of producing said enzyme by cloning of said gene and a gene which has a certain degree of homology with said gene. Regarding the homology, a gene having a homology of at least 50% or more, preferably a gene having a homology of 80% or more and more preferably a gene having a homology of 95% or more can be exemplified. The following polynucleotide (DNA or RNA) is desirable as the gene which encodes the enzyme of the present invention.

A polynucleotide which comprises a polynucleotide being selected from the following polynucleotides (a) to (g) and encoding a polypeptide having the activity to act upon a disaccharide glycoside and thereby release saccharides from said disaccharide glycoside in disaccharide unit;



(a) a polynucleotide which encodes a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing,

(b) a polynucleotide which encodes a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing, wherein one or more amino acid residues of the amino acid sequence are modified by at least one of deletion, addition, insertion and substitution,

(c) a polynucleotide which has the nucleotide sequence of SEQ ID NO: 7 shown in the Sequence Listing,

(d) a polynucleotide which has the nucleotide sequence of SEQ ID NO: 7 shown in the Sequence Listing, wherein one or more bases of the nucleotide sequence are modified by at least one of deletion, addition, insertion and substitution,

(e) a gene which hybridizes with any one of the aforementioned polynucleotides (a) to (d) under a stringent condition,

(f) a polynucleotide which has homology with any one of the aforementioned polynucleotides (a) to (d), and

(g) a polynucleotide which is degenerate with respect to any one of the aforementioned polynucleotides (a) to (f).

The gene which encodes the enzyme of the present invention can be prepared from the aforementioned

microorganism capable of producing the enzyme of the present invention by carrying out cloning of said gene in the following manner. Firstly, the enzyme of the present invention is isolated and purified from a microorganism capable of producing the enzyme of the present invention by the aforementioned method and information on its partial amino acid sequence is obtained.

Regarding the determination method of a partial amino acid sequence, it is effective to carry out a method in which purified enzyme is directly applied to an amino acid sequence analyzer (such as Protein Sequenser 476A, manufactured by Applied Biosystems) by Edman degradation method [*J. Biol. Chem.*, vol. 256, pp. 7990 - 7997 (1981)], or a method in which limited hydrolysis of the enzyme is carried out using a protein hydrolase, the thus obtained peptide fragments are isolated and purified and then amino acid sequences of the thus purified peptide fragments are analyzed.

Based on the information of the thus obtained partial amino acid sequences, a gene which encodes the enzyme of the present invention is cloned. In general, the cloning is carried out making use of a PCR method or a hybridization method.

When a hybridization method is used, the method described in "Molecular Cloning, A Laboratory Manual"

(edit. by T. Maniatis et al., Cold Spring Harbor Laboratory, 1989) may be used.

When a PCR method is used, the following method may be used.

Firstly, a gene fragment of interest is obtained by carrying out PCR reaction using genomic DNA of a microorganism capable of producing the enzyme of the present invention as the template and synthetic oligonucleotide primers designed based on the information of partial amino acid sequences. The PCR method is carried out in accordance with the method described in "PCR Technology" (edit. by Erlich H.A., Stockton Press, 1989). When nucleotide sequences of the thus amplified DNA fragments are determined by a usually used method such as the dideoxy chain termination method, a sequence which corresponds to the partial amino acid sequence of the enzyme of the present invention is found in the thus determined sequences, in addition to the sequences of synthetic oligonucleotide primers, so that a part of the enzyme gene of interest of the present invention can be obtained. As a matter of course, a gene which encodes complete enzyme of the present invention can be cloned by further carrying out a cloning method such as the hybridization method using the thus obtained gene fragment as a probe.

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In the following Examples, a gene coding for the enzyme of the present invention was determined by the PCR method using *Aspergillus fumigatus* IAM 2046. Complete nucleotide sequence of the gene coding for the enzyme of the present invention originated from *Aspergillus fumigatus* is shown in the SEQ ID NO: 7, and the amino acid sequence encoded thereby was determined to be the sequence shown in the SEQ ID NO: 8. In this connection, there are countless nucleotide sequences which correspond to the amino acid sequence shown in the SEQ ID NO: 8, in addition to the nucleotide sequence shown in the SEQ ID NO: 8, and all of these sequences are included in the scope of the present invention.

The gene of interest can also be obtained by chemical synthesis based on the information of the amino acid sequence shown in the SEQ ID NO: 8 and the nucleotide sequence shown in the SEQ ID NO: 7 (cf. Gene, 60(1), 115 - 127 (1987)).

Regarding the gene of the object enzyme of the present invention, a polynucleotide which encodes a polypeptide having the amino acid sequence of SEQ ID NO: 8, wherein one or more amino acid residues of the amino acid sequence are modified by at least one of deletion, addition, insertion and substitution, a gene which hybridizes with said polynucleotide under a stringent

condition, a polynucleotide which has homology with said polynucleotide and a polynucleotide which is degenerate with respect to said polynucleotide are also included in the present invention, with the proviso that the polypeptides encoded thereby have the enzyme activity of the present invention.

The term "under stringent condition" as used herein means, for example, the following condition. That is, 6 x SSC, 1.0% blocking agent, 0.1% N-lauroylsarcosine sodium, 0.02% SDS.

By using the entire portion or a part of the enzyme gene of the present invention, whose complete nucleotide sequence has been revealed making use of *Aspergillus fumigatus* IAM2046, as a probe for hybridization, DNA fragments having high homology with the enzyme gene of the present invention shown in SEQ ID NO: 7 can be selected from genomic DNA libraries or cDNA libraries of microorganisms capable of producing other enzymes of the present invention.

The hybridization can be carried out under the aforementioned stringent condition. For example, a genomic DNA library or a cDNA library obtained from a microorganism capable of producing an enzyme of the present invention is fixed on nylon membranes, and the thus prepared nylon membranes are subjected to blocking at 65°C in a pre-



and homology. Alternatively, it is possible to determine whether the gene encodes a polypeptide which has the enzyme activity of interest by producing a polypeptide of the gene and measuring its enzyme activity.

The following method is convenient for producing a polypeptide having the enzyme activity of the present invention using the enzyme gene of the present invention.

Firstly, transformation of a host is carried out using a vector containing the object gene of the enzyme of the present invention and then culturing of the thus obtained transformant is carried out under generally used conditions, thereby allowing the strain to produce a polypeptide having the enzyme activity of the present invention.

Examples of the host to be used include microorganisms, animal cells and plant cells. Examples of the microorganisms include bacteria such as *Escherichia coli* and other bacteria belonging to the genera *Bacillus*, *Streptomyces*, and *Lactococcus*, yeasts such as those belonging to the genera *Saccharomyces*, *Pichia* and *Kluyveromyces* and filamentous fungi such as those belonging to the genera *Aspergillus*, *Penicillium*, *Trichoderma* and *Rhizopus*. Examples of the animal cells include those which unitize the baculovirus expression system.

Confirmation of the expression and expressed product can be made easily by the use of an antibody specific for the enzyme of the present invention, and the expression can also be confirmed by measuring the enzyme activity of the present invention.

As described in the foregoing, purification of the enzyme of the present invention from the transformant culture medium can be carried out by optional combination of centrifugation, UF concentration, salting out and various types of chromatography such as of ion exchange resins.

In addition, since the primary structure and gene structure of the enzyme of the present invention have been revealed by the present invention, it is possible to obtain a gene coding for the amino acid sequence wherein one or more amino acid residues of the amino acid sequence are modified by at least one of deletion, addition, insertion and substitution, by introducing random mutation or site-specific mutation using the gene of the present invention. This method renders possible preparation of a gene coding for an enzyme of the present invention which has the enzyme activity of the present invention but its properties such as optimum temperature, temperature stability, optimum pH, pH stability and substrate specificity are slightly changed, and it also renders possible production of such



enzymes of the present invention by means of genetic engineering techniques.

Examples of the method for introducing random mutation include a chemical DNA modification method in which a transition mutation is induced to convert cytosine base into uracil base by the action of sodium hydrogensulfite [*Proceedings of the National Academy of Sciences of the USA*, vol. 79, pp. 1408 - 1412 (1982)], a biochemical method in which base substitution is induced during the step of double strand formation in the presence of [ $\alpha$ -S] dNTP [*Gene*, vol. 64, pp. 313 - 319 (1988)] and a PCR method in which PCR is carried out by adding manganese to the reaction system to decrease fidelity of the nucleotide incorporation [*Analytical Biochemistry*, vol. 224, pp. 347 - 353 (1995)].

Examples of the method for introducing site-specific mutation include a method in which amber mutation is employed [gapped duplex method; *Nucleic Acids Research*, vol. 12, no. 24, pp. 9441 - 9456 (1984)], a method in which recognition sites of restriction enzymes are used [*Analytical Biochemistry*, vol. 200, pp. 81 - 88 (1992); *Gene*, vol. 102, pp. 67 - 70 (1991)], a method in which mutation of dut (dUTPase) and ung (uracil DNA glycosylase) is used [Kunkel method; *Proceedings of the National Academy of Sciences of the USA*, vol. 82, pp. 488 - 492 (1985)], a

method in which amber mutation is induced using DNA polymerase and DNA ligase [oligonucleotide-directed dual amber (ODA) method: *Gene*, vol. 152, pp. 271 - 275 (1995); JP-A-7-289262], a method in which a host introduced with a DNA repair system is used (JP-A-8-70874), a method in which a protein capable of catalyzing DNA chain exchange reaction is used (JP-A-8-140685), a method in which PCR is carried out using two different primers for mutation use to which recognition sites of restriction enzymes are added (U.S. Patent 5,512,463), a method in which PCR is carried out using a double-stranded DNA vector having an inactivated drug resistance gene and two different primers [*Gene*, vol. 103, pp. 73 - 77 (1991)] and a method in which PCR is carried out making use of amber mutation (WO 98/02535).

Also, site-specific mutation can be introduced easily by the use of commercially available kits. Examples of such kits include Mutan<sup>®</sup>-G (manufactured by Takara Shuzo) in which the gapped duplex method is used, Mutan<sup>®</sup>-K (manufactured by Takara Shuzo) in which the Kunkel method is used, Mutan<sup>®</sup>-Express Km (manufactured by Takara Shuzo) in which the ODA method is used and QuickChange<sup>®</sup> Site-Directed Mutagenesis Kit (manufactured by STRATAGENE) in which primers for mutation use and *Pyrococcus furiosus* DNA polymerase are used, as well as TaKaRa LA PCR in vitro Mutagenesis Kit (manufactured by Takara Shuzo) and Mutan<sup>®</sup>-

Super Express Km (manufactured by Takara Shuzo) as kits in which PCR is used.

Thus, the primary structure and gene structure of the enzyme of the present invention provided by the present invention render possible production of an inexpensive and high purity polypeptide having the enzyme activity of the present invention by means of genetic engineering techniques.

In this connection, various literature and references are cited in the specification, and all of them are incorporated herein by references.

Next, various applications of the enzyme composition of the present invention are described.

Diglycosidase can be used for the improvement of various components such as aromas, colors and physiologically active contents of plant materials and for adjusting extraction efficiency of these components. In consequence, it can be used in the production of food and drinks having increased aromas and of spices, perfumes and liquid scents having increased aromas, and it also can be used for the early stage release of unfavorable odor by optionally using it during a step of the just described productions. Regarding the colors, it can be used for the improvement of hues of plant materials, food and drinks, development of colors and production of pigments.

In addition, similar to the case of aromatic components, it can be used for the degradation and removal of pigment precursors which are not desirable in view of qualities, and regarding the physiological activities, it can be used for the increase of pharmacological components and useful physiologically active components of crude drugs, herbs and other plant components or degradation and removal of undesirable components.

That is, it is possible to produce the aforementioned actions by allowing the diglycosidase of the present invention to act upon various disaccharide glycoside components.

In addition, the diglycosidase of the present invention may be administered with the aforementioned physiologically active substance, etc. after mixing or without mixing but by simultaneously or with a short interval administration, in order for the physiologically active substance to be absorbed efficiently into the body, etc.

Examples of the materials containing disaccharide glycosides to be treated by the present invention include those which undergo the action of diglycosidase, such as foods, cosmetics, medicaments, quasi drugs, agricultural chemicals and feeds, more illustratively, it can also be applied to the production of industrial products having

various aromas, such as foods, toiletries, woodworks and mats produced from plant materials.

Food articles having aromatic components can be exemplified as materials to which the diglycosidase of the present invention is preferably applied. As illustrative examples, it can be used in the so-called "wilting" step during the production of oolong tea and jasmine tea and for the improvement of aromas of black tea (for tea pack by CTC method) and wine. It can also be used for the maintenance of aromas of cosmetics and liquid scents and improvement of aromas and pharmacological effects of medicaments.

The diglycosidase of the present invention is also useful in the production of pigments. For example, extraction of alizarine dye from *Rubia tinctorum* L. ruberythric acid can be carried out more efficiently than the conventional method by the use of the enzyme.

Also, it is possible to produce precursors of disaccharide components such as an aroma, a pigment, a physiologically active component and primeverose making use of the action of diglycosidase. Improvement of the stability and keeping quality of these components, their detoxication and modification of pharmacological components for DDS can be expected by their glycosylation.

In addition, diglycosidase can degrade modified glucosides such as acetylglucoside, malonylglucoside,



Also, the enzyme of the present invention can be used for increasing concentration of an aroma, pigment or physiologically active component which is present in plants. That is, since plants contain precursors of these components, an aroma, pigment or physiologically active component in a plant can be increased by cultivating the plant with adding an effective amount of the diglycosidase of the present invention (including transgenic method) under such conditions that the precursor in said plant can be hydrolyzed. In addition, the formation period of an aroma, pigment or physiologically active component can be controlled making use of the enzyme composition of the present invention.

In this connection, it is possible to synthesize various types of glycoside by making use of reverse reaction of the diglycosidase of the invention.

#### Best Mode for Carrying Out the Invention

The invention is described further in detail in the following with reference to examples but, as a matter of course, the invention is not limited to the following examples without departing from its scope. Unless otherwise noted, the term % as used herein means w/v %.

#### Example 1

Each of *Aspergillus niger* IFO 4407 and *Aspergillus niger* IAM 2020 was cultured overnight at 30°C on a shaker





diglycosidase activity was 0.106 unit/ml and  $\beta$ -glucosidase activity was 0.320 unit/ml.

### Example 3

Using *Aspergillus fumigatus* IAM 2046, effects of inducers on the production of diglycosidase were examined. *Aspergillus fumigatus* IAM 2046 was cultured for 6 days in a culture medium (2% soybean flour, 0.3% sodium chloride, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3% soluble starch) supplemented with 0.1% of each of various saccharides, and the diglycosidase activity was measured. The results are shown in Table 3.

Table 3

Inducers	Inducing ability (%)
Not added	100
Isomaltose	145
Maltotriose	171
Maltose	136
Gentose	235
Gentiobiose	211
Gentio-oligosaccharide	180
Sucrose	116
Trehalose	113
Glucose	164
Galactose	125
Fructose	143
Rhamnose	129
Tulbose	116
Maltitol	142
Arabitol	112
Galactitol	142
Glucosamine hydrochloride	157

As is evident from the above table, the producing ability of diglycosidase was increased by various saccharides. Particularly, markedly high inducing ability was found in gentose, gentiobiose and gentio-oligosaccharide.

In addition, similar effects were also found when *Aspergillus niger* IFO 4407 or *Aspergillus niger* IAM 2020 was used.

#### Example 4

Each of the crude enzyme solutions obtained in Examples 1 and 2 was concentrated using an ultrafiltration membrane having a molecular weight cutoff of 6,000. Next, 1 ml of the concentrated solution was mixed with 1 ml of a 5 mg/ml pNP-primeveroside solution which had been prepared using 20 mM phosphate buffer (pH 6.0), and the mixture was incubated at 37°C. Samples were collected 1, 2, 4, 24 and 48 hours thereafter to confirm release of primeverose by a thin layer chromatography (TLC).

As a result, a spot was observed by TLC at the same position of a disaccharide primeverose in all of the culture media of two *Aspergillus niger* strains described in Example 1 and *Aspergillus fumigatus* described in Example 2. Such a spot was not observed in samples in which the crude enzyme concentration solutions were subjected to the same test after their heat treatment (100°C for 10 minutes).

Thus, the presence of an enzyme capable of releasing primeverose in disaccharide unit from pNP-primeveroside was found in the crude enzyme concentration solutions.

#### Example 5

#### Screening of diglycosidase in various microorganisms

##### a) Preparation of enzyme samples

Each microorganism to be subjected to screening was pre-cultured and main cultured. In the case of liquid culturing, the obtained culture broth was centrifuged at  $10,000 \text{ min}^{-1}$  for 10 minutes and the resulting supernatant was used as the enzyme sample. In the case of solid culturing, the medium after completion of the culturing was extracted with water and the resulting extract was used as the enzyme sample.

In this connection, intracellular enzyme was also examined in the case of bacteria. In that case, the culture broth was centrifuged, and the thus obtained cells as the precipitate were washed with physiological saline, suspended in 10 times amount of 10 mM phosphate buffer (pH 7.0) based on the cell weight and then treated with ultrasonic wave to disrupt the cells. The disrupted suspension was centrifuged at  $12,000 \text{ min}^{-1}$  for 20 minutes and the resulting supernatant was used as the intracellular enzyme sample.

Media and culture conditions regarding the culturing are shown in Tables 1 to 5.

Table 1 Liquid culturing of mold and yeast

Pre-culture: Medium composition

Yeast extract (DIFCO)	0.2%
Peptone (DIFCO)	0.5%
Glucose (Katayama Chemical)	2.0%
Potassium dihydrogenphosphate (Kanto Chemical)	0.1%
Magnesium sulfate heptahydrate (Katayama Chemical)	0.05%

The above composition was dissolved in purified water and adjusted to pH 5.7 with 1 M hydrochloric acid and 1 M sodium hydroxide. The medium was dispensed in 100 ml portions into Sakaguchi flasks and then sterilized at 121°C for 20 minutes under 1 atmospheric pressure.

Pre-culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with one loopful inoculum from a slant culture, for 1 day or more and at 30°C. Regarding yeast strains, the temperature condition was set to 25°C.

Main culture: Medium composition

Soya flower A (Nisshin)	2.0%
Sodium chloride (Kanto Chemical)	0.3%
Dipotassium hydrogenphosphate (Kanto Chemical)	0.1%

Magnesium sulfate heptahydrate (Katayama Chemical)	0.05%
Soluble starch (Wako Pure Chemical)	3.0%
Gentose #80 (Nihon Shokuhin Kako)	0.5%

The above composition was dissolved in purified water and adjusted to pH 5.6 with 1 M hydrochloric acid and 1 M sodium hydroxide. The medium was dispensed in 100 ml portions into Sakaguchi flasks and then sterilized at 121°C for 20 minutes under 1 atmospheric pressure.

Main culture: Culture conditions

Table 2 Solid culturing of mold and yeast

High starch bran  
(B Ohgi, Nippon Flower Milling) 8.3%

Pre-culture: Culture conditions

The culturing was carried out at a shaking speed of 300 min<sup>-1</sup>, with one loopful inoculum from a slant culture, for 1 to 2 days and at 30°C.

Main culture: Medium composition

A 5.0 g portion of bran was suspended in 1.5 ml of purified water, dispensed into 100 ml capacity conical flasks and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Main culture: Culture conditions

The culturing was carried out with 1 ml inoculum from the pre-culture broth, for 3 days and at 30°C.

Extraction

By adding 90 ml of tap water, extracted overnight at 8°C or below.

Table 3 Culturing of bacteria and actinomycetes

Pre-culture: Medium composition

Tryptic soy broth (DIFCO)

BACTO Tryptone	1.7%	} pH 7.3 ± 0.2
BACTO Soytone	0.3%	
BACTO Dextrose	0.2%	
Sodium chloride	0.5%	
Dipotassium hydrogenphosphate	0.25%	

The above composition was dissolved in purified water, and the medium was dispensed in 100 ml portions into

Sakaguchi flasks and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Pre-culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with one loopful inoculum from a slant culture, for 1 day or more and at 30°C.

Main culture: Medium composition

Polypeptone (Japan Pharmaceutical)	1.0%
Yeast extract (DIFCO)	0.25%
Ammonium sulfate (Wako Pure Chemical)	0.1%
Dipotassium hydrogenphosphate (Kanto Chemical)	0.05%
Magnesium sulfate heptahydrate (Katayama Chemical)	0.025%
Calcium chloride (Wako Pure Chemical)	0.0001%
Adekanol LG126 (Asahi Denka)	0.001%
Gentose #80 (Nihon Shokuhin Kako)	0.5%

The above composition was dissolved in purified water and adjusted to pH 7.0 with 1 M hydrochloric acid and 1 M sodium hydroxide. The medium was dispensed in 100 ml portions into Sakaguchi flasks and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

The culturing was carried out using the medium both in the presence and absence of gentose #80.

Main culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with 1 ml inoculum from the pre-culture broth, for 5 days and at 30°C.

Table 4 Culturing of *Penicillium multicolor*

Pre-culture: Medium composition

Defatted soybean "Soypro" (Hohnen Oil)	2.0%
Glucose (Katayama Chemical)	3.0%
Potassium dihydrogenphosphate (Kanto Chemical)	0.5%
Ammonium sulfate (Wako Pure Chemical)	0.4%
Dry yeast	0.3%
Adekanol (Asahi Denka)	0.05%

The above composition was dissolved in purified water, and the medium was dispensed in 100 ml portions into Sakaguchi flasks and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Pre-culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with one loopful inoculum from a slant culture, for 5 days or more and at 27°C.

Main culture: Medium composition

Gentose #80 (Nihon Shokuhin Kako)	3.0%
Potassium dihydrogenphosphate (Kanto Chemical)	2.0%
Ammonium sulfate (Wako Pure Chemical)	1.0%



Meast P1G (Asahi Beer Food) 3.13%

Adekanol LG126 (Asahi Denka) 0.05%

The above composition was dissolved in purified water, and the medium was dispensed in 100 ml portions into Sakaguchi flasks and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Main culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with 1 ml inoculum from the pre-culture broth, for 6 days and at 27°C.

Table 5 Culturing of the genus *Corynebacterium*

Pre-culture: Medium composition

Glucose	0.2%
Yeast extract	0.1%
Ammonium nitrate	0.4%
Potassium dihydrogenphosphate	0.15%
Sodium hydrogenphosphate dodecahydrate	0.15%
Magnesium sulfate heptahydrate	0.02%
Ferrous sulfate heptahydrate	0.0001%
Calcium chloride dihydrate	0.001%

The above composition was dissolved in purified water and adjusted to pH 7.0 with 1 M hydrochloric acid and 1 M sodium hydroxide. The medium was dispensed in 10 ml portions into culture test tubes and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Pre-culture: Culture conditions

The culturing was carried out at a shaking speed of 300 min<sup>-1</sup>, with one loopful inoculum from a slant culture, for 2 days and at 30°C.

Main culture: Medium composition

Eugenyl- $\beta$ -primeveroside	0.2%
Yeast extract	0.1%
Ammonium nitrate	0.4%
Potassium dihydrogenphosphate	0.15%
Sodium hydrogenphosphate dodecahydrate	0.15%
Magnesium sulfate heptahydrate	0.02%
Ferrous sulfate heptahydrate	0.0001%
Calcium chloride dihydrate	0.001%

The above composition was dissolved in purified water and adjusted to pH 7.0 with 1 M hydrochloric acid and 1 M sodium hydroxide. The medium was dispensed in 10 ml portions into culture test tubes and then sterilized at 121°C for 20 minutes under one atmospheric pressure.

Main culture: Culture conditions

The culturing was carried out at a shaking speed of 140 min<sup>-1</sup>, with 1 ml inoculum from the pre-culture broth, for 1 day and at 30°C.

b) Preparation of substrate solution

PNP- $\beta$ -primeveroside was dissolved in 20 mM acetate buffer (pH 5.5) to a concentration of 5 mg/ml and used as a

substrate solution A. Eugenyl- $\beta$ -primeveroside was dissolved in 20 mM acetate buffer (pH 5.5) to a concentration of 10 mg/ml and used as a substrate solution B.

c) Enzyme reaction

A 100  $\mu$ l portion of the substrate solution A was put into a micro-centrifugation tube and mixed with 100  $\mu$ l of each enzyme sample to carry out 96 hours of the enzyme reaction in a water bath of 37°C. When the reaction reached the intended time, the reaction solution was treated at 100°C for 10 minutes to stop the enzyme reaction. This was used as the enzyme reaction-completed solution.

As a comparative control of the enzyme sample, the same enzyme sample was treated at 100°C for 10 minutes before the enzyme reaction and subjected to the same reaction. The enzyme reaction was carried out on each of the substrate solutions A and B. When the substrate solution B was used, the reaction was carried out in the same manner as the case of the substrate solution A.

d) Thin layer chromatography

20  $\mu$ l of the enzyme reaction-completed solution was spotted on a thin layer of silica gel (Silica gel 60 F254 [1.05554], Merck) and dried. This was developed twice with a developing solvent prepared by mixing ethyl acetate,

acetic acid and purified water at a ratio of 3:1:1. After completion of the development, the thin layer was air-dried. Thereafter, a color developing reagent prepared by mixing sulfuric acid and methanol at a ratio of 20:80 was sprayed all over the thin layer after completion of the development, and the color was developed at 105°C for about 10 minutes.

An enzyme sample by which the spot of primeverose was appeared on the thin layer after the color development was judged that the primeverosidase was present therein, and this producer strain was judged as a diglycosidase producing strain.

e) Results of the screening of diglycosidase producing strains

A summary of the diglycosidase producing strains found by the above evaluation method is shown in Table 6.

Table 6 Strains in which the diglycosidase production was found

Microorganisms	Strain names
Mold	<i>Aspergillus oryzae</i> IAM 2769
	<i>Aspergillus niger</i>
	IAM 2020
	IFO 4091
	IFO 9455
	IAM 2107
	<i>Aspergillus aculeatus</i>
	<i>Penicillium rugulosum</i> IFO 7242
	<i>Penicillium lilacinum</i> IFO 5350
	<i>Penicillium decumbence</i> IFO 31297
	<i>Penicillium multicolor</i> IAM 7153
	<i>Rhizopus oryzae</i> JCM 5560
	<i>Rhizomucor pusillus</i> IAM 6122
	<i>Rhizomucor miehei</i> IFO 9740
Yeast	<i>Talaromyces emersonii</i> IFO 9747
	<i>Mortierella vinacea</i> IFO 7875
Bacteria	<i>Cryptococcus albidus</i> IAM 12205
Actinomycetes	<i>Microbacterium arborescens</i> JCM 5884
	<i>Corynebacterium ammoniagenes</i> IFO 12072
	<i>Corynebacterium ammoniagenes</i> IFO 12612
	<i>Corynebacterium glutamicum</i> IFO 1318
	<i>Actinoplanes missouriensis</i> JCM 3121

#### Example 6

#### Purification of diglycosidase derived from *Aspergillus fumigatus*

As the pre-culture, *Aspergillus fumigatus* IAM 2046 was inoculated into a glucose-peptone medium (0.2% yeast extract, 0.5% peptone, 2% glucose, 0.1% potassium dihydrogenphosphate, 0.05% magnesium sulfate, pH 5.7) and cultured at 30°C for 24 hours on a shaker. The pre-culture

broth was inoculated in an inoculum size of 1% into the main culture medium (2% Soya flower, 0.3% sodium chloride, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, 3% soluble starch, 1% gentose #80, pH 5.6) and cultured at 30°C for 6 days on a shaker.

Cells were removed from the culture broth by filter paper filtration, and 8,600 ml of the resulting filtrate was concentrated to 710 ml using an ultrafiltration membrane of 6,000 molecular weight cutoff (AIP-1010, mfd. by Asahi Chemical Industry). A 200 ml portion of the concentrated solution was centrifuged at 4°C and at 15,000 rpm for 10 minutes, and 192 ml of the supernatant was mixed with 55.9 g of ammonium sulfate (50% saturation) and stirred overnight at 4°C. This was centrifuged at 4°C and at 15,000 rpm for 10 minutes, and the thus obtained precipitate was dissolved in 10 ml of 20% saturation ammonium sulfate/20 mM phosphate buffer (pH 6.0) and centrifuged at 4°C and at 15,000 rpm for 10 minutes to recover the supernatant. A 9.5 ml portion of the supernatant was applied to a Phenyl Sepharose column (16 x 100 mm, mfd. by Pharmacia) which had been equilibrated with 20% saturation ammonium sulfate/20 mM phosphate buffer (pH 6.0), and the adsorbed protein was released by an ammonium sulfate linear density gradient of from 20% to 0%. The active peaks were recovered, buffer-exchanged to 25 mM

triethanolamine buffer (pH 8.3) using 10DG column (mfd. by BIO-RAD), applied to an anion exchange Mono-P column (5 x 200 mm, mfd. by Pharmacia) which had been equilibrated with 25 mM triethanolamine buffer (pH 8.3) and then eluted with Polybuffer (pH 5.0, mfd. by Pharmacia), and the thus adsorbed protein was released by a pH linear density gradient of from pH 8.3 to pH 5.0 to obtain a purified enzyme preparation of diglycosidase. An SDS-PAGE analysis confirmed that the enzyme was purified as a single band of 47 kDa. Also, when it was treated with Endoglycosidase H (mfd. by BOEHRINGER MANNHEIM), changes in the band size were not found.

#### Example 7

#### Physicochemical properties of diglycosidase derived from *Aspergillus fumigatus*

Its optimum pH was measured in the following manner. A 400  $\mu$ l portion of 2 mM pNP-primeveroside solution which had been adjusted to a respective pH value of from pH 2 to 5 with 20 mM secondary citric acid-HCl buffer was incubated at 37°C for 5 minutes. Next, this was mixed with 90  $\mu$ l of the enzyme solution to carry out the reaction at 37°C for 20 minutes. The reaction was stopped by adding 500  $\mu$ l of 0.5 M sodium carbonate solution, and the activity measurement was carried out by measuring the absorbance at 420 nm. As a result, it was found that its optimum pH was

from 2.5 to 3.0. It was found that it shows sufficient activity at pH 3 which is more lower pH value than those of plant-derived enzymes having similar activity.

Its optimum temperature was measured in the following manner. A 400  $\mu$ l portion of 2 mM pNP-primeveroside solution prepared using 20 mM secondary citric acid-HCl buffer (pH 2.5) was mixed with 90  $\mu$ l of the enzyme solution to carry out the reaction at 30 to 65°C for 20 minutes. The reaction was stopped by adding 500  $\mu$ l of 0.5 M sodium carbonate solution, and the activity was determined by measuring the absorbance at 420 nm. It was found that sufficient activity is maintained, because diglycosidase derived from *Aspergillus fumigatus* has 80% of the activity even at 60°C, in comparison with the plant-derived enzymes having similar activity.

Its pH stability was measured in the following manner. The purified enzyme preparation was diluted 100 times with each of secondary citrate buffer of from pH 2 to 5, phosphate buffer of from pH 6 to 8 or glycine NaCl-NaOH buffer of from pH 7 to 10 and treated at 37°C for 1 hour, and then a 90  $\mu$ l portion thereof was mixed with 400  $\mu$ l of 2 mM pNP-primeveroside solution (pH 2.5) which had been incubated at 37°C for 5 minutes, and the reaction was carried out at 37°C for 20 minutes. The reaction was stopped by adding 500  $\mu$ l of 0.5 M sodium carbonate



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10022024

solution, and the activity measurement was carried out by measuring the absorbance at 420 nm to calculate the residual activity. As a result, its pH stability was 100% at pH 8 and it was stable within a range of from pH 3 to 8. It was found that this enzyme is stable within broader pH range in comparison with plant-derived enzymes which have similar activity and are stable at pH 4 to 7.

Thermal stability of the purified preparation was examined by diluting it 100 times with 20 mM glycine NaCl-NaOH buffer (pH 8), treating the dilution at each temperature of from 30 to 55°C for 1 hour and then measuring the residual activity. As a result, the activity was stable at a temperature of 50°C or below. It was found that this enzyme was stable broader range of temperature, in comparison with plant-derived enzymes which have similar activity and are stable at 45°C or below.

Physicochemical properties of diglycosidase derived from other microorganisms

Diglycosidase samples, the production of which had been confirmed by the same method as described in Example 5, were examined for physicochemical properties. As a result, it was found that diglycosidase has an activity enough for practical use at pH 3 or less because of its optimum pH ranging from 3 to 6, that it has an activity enough for practical use at 50°C or more because of its

optimum temperature ranging from 30°C to 60°C, and that it is stable at pH 3 to 8 and at 50°C or less. Thus, diglycosidase derived from microorganisms can be used in relatively broad ranges of pH and temperature in comparison with similar enzymes derived from plants and is superior thereto in stability.

#### Example 8

#### Isolation of gene coding for the diglycosidase derived from *Aspergillus fumigatus*

Unless otherwise noted, gene manipulation techniques employed herein were carried out in accordance with a textbook (e.g., Molecular Cloning 2nd ed., Cold Spring Harbor Laboratory Press, 1989).

##### a) Isolation of chromosomal DNA

*Aspergillus fumigatus* IAM 2046 was inoculated into a glucose-peptone medium (0.2% yeast extract, 0.5% peptone, 2% glucose, 0.1% potassium dihydrogenphosphate, 0.05% magnesium sulfate, pH 5.7) and cultured at 30°C for 3 days on a shaker.

In accordance with the method of Michael J. Hynes (*Molecular and Cellular Biology*, 1983, Vol. 3, No. 8, 1430 - 1439), 0.2 ml of chromosomal DNA having a concentration of 12.6 mg/ml was obtained from 300 ml of the culture broth.

##### b) Determination of partial amino acid sequence

The purified enzyme preparation of diglycosidase obtained in the Example was applied to a protein sequencer (mfd. by Hewlett Packard) to determine the 22 residue N-terminal amino acid sequence shown in SEQ ID NO: 1. Next, the purified enzyme preparation of diglycosidase obtained in the Example was subjected to reductive carboxymethylation and then digested with lysyl endopeptidase. The thus obtained digest was applied to a reverse phase liquid chromatography, and one of the digested peptide fractions was applied to the protein sequencer to determine the 22 residue internal amino acid sequence shown in SEQ ID NO: 2.

SEQ ID NO: 1

Ala-Ala-Ser-Ala-Ser-Ala-Tyr-Cys-Ser-Asn-Ser-Ala-Gly-Asn-Tyr-Lys-Leu-Ser-Ser-Ile-Ala-Ala

SEQ ID NO: 2

Leu-Met-Thr-Pro-Ala-Gly-Ala-Asn-Phe-Ala-Leu-Met-Arg-His-Thr-Ile-Gly-Ala-Ser-Asp-Leu-Ser

c) Preparation of DNA probe by PCR

Based on the N-terminal amino acid sequence and internal amino acid sequence, the following four mixed oligonucleotides were synthesized by a DNA synthesizer and used as PCR primers.

SEQ ID NO: 3

Sense primer:

5'-ACGAATTCAA (TC) (TA) (CG) IGC (TCAG) GGIAA (TC) TA (TC) AA-3'

SEQ ID NO: 4

Sense primer:

5'-CGGAATTCTA (TC) TG (TC) (TA) (CG) IAA (TC) (TA) (CG) IGC (TCAG) GG-3'

SEQ ID NO: 5

Antisense primer:

5'-TCAAGCTTGC (AG) AA (AG) TTIGC (TCAG) CCIGC (TCAG) GG-3'

SEQ ID NO: 6

Antisense primer:

5'-AGAAGCTTGCICC (TAG) ATIGT (AG) TG (TCAG) C (TG) CAT

Using these primers and the *Aspergillus fumigatus* chromosomal DNA as the template, PCR reaction was carried out under the following conditions using GeneAmp PCR System 9600 (Perkin Elmer).

<PCR reaction solution>

10 x PCR reaction buffer (Perkin Elmer)	10 µl
dNTP mixed solution (each 2 mM, Perkin Elmer)	
	10 µl
25 mM MgCl <sub>2</sub> (Perkin Elmer)	6 µl
chromosomal DNA solution (100 µg/ml)	1 µl
40 µM sense primer	2.5 µl
40 µM antisense primer	2.5 µl
sterilized water	67.5 µl
Amplitaq Gold (5 U/µl, Perkin Elmer)	0.5 µl

<PCR reaction conditions>

Stage 1: denaturation (95°C, 9 minutes) 1 cycle  
Stage 2: denaturation (94°C, 45 seconds) 30 cycles  
annealing (55°C, 1 minute)  
elongation (72°C, 2 minutes)  
Stage 3: elongation (72°C, 10 minutes) 1 cycle

When the thus obtained DNA fragment of about 0.27 kbp was cloned into pUC19 (TOYOBO) and then its nucleotide sequence was examined, a nucleotide sequence coding for the partial amino acid sequence described in the foregoing was found between just after the sense primer and just before the antisense primer. This DNA fragment was used as the DNA probe for the gene cloning.

d) Preparation of gene library

By recovering total RNA from *Aspergillus fumigatus*, Poly(A) RNA was prepared using Poly(A)Quick mRNA Isolation Kit (mfd. by STRATAGENE). Next, cDNA was synthesized using ZAP-cDNA Synthesis Kit (mfd. by STRATAGENE), ligated to λZAP II vector (mfd. by STRATAGENE) and then subjected to packaging using Gigapack III Gold (mfd. by STRATAGENE) to obtain a gene library.

e) Screening of gene library

The 0.27 kbp DNA fragment obtained in the aforementioned step c) was labeled using DIG-High Prime (mfd. by BOEHRINGER MANNHEIM). Using this as the DNA probe, the gene library obtained in the step d) was

screened by plaque hybridization. After recovering phage particles from the thus obtained positive plaques, a plasmid pAFPri containing a cDNA of about 1.7 kbp was obtained by the *in vivo* excision method in accordance with the instruction of STRATAGENE.

f) determination of nucleotide sequence

A nucleotide sequence coding for the diglycosidase is shown in SEQ ID NO: 7. Also, an amino acid sequence encoded by the SEQ ID NO: 7 is shown in SEQ ID NO: 8. Since the N-terminal amino acid sequence (SEQ ID NO: 1) and internal amino acid sequence (SEQ ID NO: 2) determined in the step b) were found in this amino acid sequence, it was confirmed that this DNA fragment is a diglycosidase gene fragment.

SEQ ID NO: 7

gcccctctg ctctggctta ctgttccaac tcggccggca actacaagct gtccctccatc 60  
gcagctccgg ttcaaggggc cggaaacccc ggctcggaaat cgacctggca attgaccgtt 120  
gacgacactt cgtccgttca caaacagacg atagttaggt tcggtgctgc tgtcactgat 180  
gccacggtca cctcgttcaa cactttgtcc gcctccgtgc tgcaagactt gctcaataaa 240  
ctgatgacac ctgccggggc gaactttgct ttgatgcgac atactattgg ggcttcggat 300  
ctgtccgggtg acccagccta cactgtacgat gacaatgggt ggaagacgga tccgtcactg 360  
tcgggattca acctggggga ccgcggaacg gctatggcca agatgttggc aacaatgaag 420  
tctctgcagc ccaacctcaa gatcctcggc tctccctgga gtgcaccagg atggatgaag 480  
ctgaacgggg tccttgatgg caatacgaac aacaacaact tgaacgatgg atacctaacc 540  
agtgggggaa ccggtagtac ggggtatgcc agtcaattcg cgcagtactt tgtcaagtac 600  
attcaggcct ataagaatct cgggtgtcac gtgcagcga ttaccatcca gaacgagccg 660  
ctgttcagct cagcgggcta tcccacatg tatgtctacg attatgagtc ggcacagctg 720  
atccagaact acatcggccc cgtctttgcc agcgcggggc tagatacga aatctgggct 780  
tatgaccaca acacagatgt cccgtcgtac cccagactg tccttaacca ggccgggtcag 840  
tacgtcaagt cgggtggcctg gcactgctac gctcccaacg tcgactggac cgtgctcagc 900  
cagttccaca acacaaaccc tggagtgaag caatatatga ccgagtgtg gactccagca 960  
tctggcgcat ggcatcaggc ggccggacttc accatgggtc ccttcagaa ctgggcctcg 1020  
ggagtggcag catggactct gggaaccaac gtcaggatg gtcgcgatct gtccactggc 1080  
ggctgcgcga catgtcaagg cttggtgacc atcaacaacg gaggatacac gotcaacacc 1140  
gcatactaca tgatggcgca attcagcaag ttcatgccgc ctggtgcgat tgtgctcaat 1200  
ggcagtgcca gctacacgta ctctggcgga ggccggtatcc agtccgtggc ttccottgaat 1260  
cccgatggaa ccgcactgt ggttattgaa aacacttttg gcaatgatgt ctatgtgact 1320  
gtcactatga agagcgggca gaagtggagt gggaacgccc ctagccaatc cgtgactacc 1380  
tgggttcttc catctgcttg a

1401

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SEQ ID NO: 8

Ala Ala Ser Ala Ser Ala Tyr Cys Ser Asn Ser Ala Gly Asn Tyr Lys  
1 5 10 15  
Leu Ser Ser Ile Ala Ala Pro Val Gln Gly Ala Gly Asn Pro Gly Ser  
20 25 30  
Glu Ser Thr Trp Gln Leu Thr Val Asp Asp Thr Ser Ser Gly His Lys  
35 40 45  
Gln Thr Ile Val Gly Phe Gly Ala Ala Val Thr Asp Ala Thr Val Thr  
50 55 60  
Ser Phe Asn Thr Leu Ser Ala Ser Val Leu Gln Asp Leu Leu Asn Lys  
65 70 75 80  
Leu Met Thr Pro Ala Gly Ala Asn Phe Ala Leu Met Arg His Thr Ile  
85 90 95  
Gly Ala Ser Asp Leu Ser Gly Asp Pro Ala Tyr Thr Tyr Asp Asp Asn  
100 105 110  
Gly Gly Lys Ala Asp Pro Ser Leu Ser Gly Phe Asn Leu Gly Asp Arg  
115 120 125  
Gly Thr Ala Met Ala Lys Met Leu Ala Thr Met Lys Ser Leu Gln Pro  
130 135 140  
Asn Leu Lys Ile Leu Gly Ser Pro Trp Ser Ala Pro Gly Trp Met Lys  
145 150 155 160  
Leu Asn Gly Val Leu Asp Gly Asn Thr Asn Asn Asn Leu Asn Asp  
165 170 175  
Gly Tyr Leu Thr Ser Gly Gly Thr Gly Ser Thr Gly Tyr Ala Ser Gln  
180 185 190  
Phe Ala Gln Tyr Phe Val Lys Tyr Ile Gln Ala Tyr Lys Asn Leu Gly  
195 200 205  
Ala His Val Asp Ala Ile Thr Ile Gln Asn Glu Pro Leu Phe Ser Ser  
210 215 220  
Ala Gly Tyr Pro Thr Met Tyr Val Tyr Asp Tyr Glu Ser Ala Gln Leu  
225 230 235 240  
Ile Gln Asn Tyr Ile Gly Pro Ala Leu Ala Ser Ala Gly Leu Asp Thr  
245 250 255  
Glu Ile Trp Ala Tyr Asp His Asn Thr Asp Val Pro Ser Tyr Pro Gln  
260 265 270  
Thr Val Leu Asn Gln Ala Gly Gln Tyr Val Lys Ser Val Ala Trp His  
275 280 285

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Cys Tyr Ala Pro Asn Val Asp Trp Thr Val Leu Ser Gln Phe His Asn  
 290 295 300  
 Thr Asn Pro Gly Val Lys Gln Tyr Met Thr Glu Cys Trp Thr Pro Ala  
 305 310 315  
 Ser Gly Ala Trp His Gln Ala Ala Asp Phe Thr Met Gly Pro Leu Gln  
 325 330 335  
 Asn Trp Ala Ser Gly Val Ala Ala Trp Thr Leu Gly Thr Asn Ala Gln  
 340 345 350  
 Asp Gly Pro His Leu Ser Thr Gly Gly Cys Ala Thr Cys Gln Gly Leu  
 355 360 365  
 Val Thr Ile Asn Asn Gly Gly Tyr Thr Leu Asn Thr Ala Tyr Tyr Met  
 370 375 380  
 Met Ala Gln Phe Ser Lys Phe Met Pro Pro Gly Ala Ile Val Leu Asn  
 385 390 395  
 Gly Ser Gly Ser Tyr Thr Tyr Ser Gly Gly Gly Ile Gln Ser Val  
 405 410 415  
 Ala Ser Leu Asn Pro Asp Gly Thr Arg Thr Val Val Ile Glu Asn Thr  
 420 425 430  
 Phe Gly Asn Asp Val Tyr Val Thr Val Thr Met Lys Ser Gly Gln Lys  
 435 440 445  
 Trp Ser Gly Asn Ala Pro Ser Gln Ser Val Thr Thr Trp Val Leu Pro  
 450 455 460  
 Ser Ala  
 465

09806413 033001

The open reading frame of this gene is shown in SEQ ID NO: 9. As shown in SEQ ID NO: 10, the entire portion is coded as a preprotein of 488 amino acids, of which the N-terminal 22 residues are assumed to be the pre-region and the remaining 466 residues correspond to the mature protein (cf. SEQ ID NO: 8).

The invention is not only particularly limited to a polypeptide having an activity to act upon a disaccharide glycoside to release saccharides from the disaccharide glycoside in disaccharide unit and a nucleotide which encodes the same, but also includes a more longer polypeptide comprising the former polypeptide (e.g., precursor) and a nucleotide which encodes the same.

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SEQ ID NO: 9

ggcgacacca gaaagcaacc aagagcacga caccgactta tttctctttg aca atg 56  
Met

cgt ata tct gtc ggt gct ctg ctt ggc ttg aca gcc ctg agt cat gcc 104  
Arg Ile Ser Val Gly Ala Leu Leu Gly Leu Thr Ala Leu Ser His Ala  
-20 -15 -10

aca aca gag aaa cga gcc gcc tct gct tgg gct tac tgt tcc aac tgg 152  
Thr Thr Glu Lys Arg Ala Ala Ser Ala Ser 5 Ala Tyr Cys Ser Asn Ser  
-5 -1 1 5 10

gcc gcc aac tac aag ctg tcc tcc atc gca gct cgg gtt caa ggg gcc 200  
Ala Gly Asn Tyr Lys Leu Ser Ser Ile Ala Ala Pro Val Gln Gly Ala  
15 20 25

gga aac ccc gcc tgg gaa tgg acc tgg caa ttg acc gtt gac gac act 248  
Gly Asn Pro Gly Ser Glu Ser Thr Trp Gln Leu Thr Val Asp Asp Thr  
30 35 40

tgg tcc ggt cac aaa cag acg ata gtt ggg ttc ggt gct gct gtc act 296  
Ser Ser Gly His Lys Gln Thr Ile Val Gly Phe Gly Ala Ala Val Thr  
45 50 55

gat gcc acg gtc acc tgg ttc aac act ttg tcc gcc tcc gtg ctg caa 344  
Asp Ala Thr Val Thr Ser Phe Asn Thr Leu Ser Ala Ser Val Leu Gln  
60 65 70 75

gac ttg ctc aat aaa ctg atg aca oct gcc ggg gcg aac ttt gct ttg 392  
Asp Leu Leu Asn Lys Leu Met Thr Pro Ala Gly Ala Asn Phe Ala Leu  
80 85 90

atg cga cat act att ggg gct tgg gat ctg tcc ggt gac cca gcc tac 440  
Met Arg His Thr Ile Gly Ala Ser Asp Leu Ser Gly Asp Pro Ala Tyr  
95 100 105

acg tac gat gac aat ggt ggg aaa gcg gat cgg tca ctg tgg gga ttc 488  
Thr Tyr Asp Asp Asn Gly Gly Lys Ala Asp Pro Ser Leu Ser Gly Phe  
110 115 120

aac ctg ggg gac cgc gga acg got atg gcc aag atg ttg gca aca atg 536  
Asn Leu Gly Asp Arg Gly Thr Ala Met Ala Lys Met Leu Ala Thr Met  
125 130 135

aag tct ctg cag ccc aac ctc aag atc ctc gcc tct ccc tgg agt gca 584  
Lys Ser Leu Gln Pro Asn Leu Lys Ile Leu Gly Ser Pro Trp Ser Ala  
140 145 150 155

cca gga tgg atg aag ctg aac ggg gtc ctt gat gcc aat acg aac aac 632  
Pro Gly Trp Met Lys Leu Asn Gly Val Leu Asp Gly Asn Thr Asn Asn  
160 165 170

aac aac ttg aac gat gga tac cta acc agt ggg gga acc ggt agt acg 680  
Asn Asn Leu Asn Asp Gly Tyr Leu Thr Ser Gly Gly Thr Gly Ser Thr  
175 180 185

09806473.033001

ggg tat gcc agt caa ttc gcg cag tac ttt gtc aag tac att cag gcc 728  
 Gly Tyr Ala Ser Gln Phe Ala Gln Tyr Phe Val Lys Tyr Ile Gln Ala  
 190 200

tat aag aat ctc ggt gct cac gtc gac gcg att acc atc cag aac gag 776  
 Tyr Lys Asn Leu Gly Ala His Val Asp Ala Ile Thr Ile Gln Asn Glu  
 205 210 215

ccg ctg ttc agc tca gcg ggc tat ccc acc atg tat gtc tac gat tat 824  
 Pro Leu Phe Ser Ser Ala Gly Tyr Pro Thr Met Tyr Val Tyr Asp Tyr  
 220 225 230 235

gag tcg gca cag ctg atc cag aac tac atc ggc ccc gct ctt gcc agc 872  
 Glu Ser Ala Gln Leu Ile Gln Asn Tyr Ile Gly Pro Ala Leu Ala Ser.  
 240 245 250

gcg ggg cta gat acg gaa atc tgg gct tat gac cac aac aca gat gtc 920  
 Ala Gly Leu Asp Thr Glu Ile Trp Ala Tyr Asp His Asn Thr Asp Val  
 255 260 265

ccg tcg tac ccc cag act gtc ctt aac cag gcc ggt cag tac gtc aag 968  
 Pro Ser Tyr Pro Gln Thr Val Leu Asn Gln Ala Gly Gln Tyr Val Lys  
 270 275 280

tcg gtg gcc tgg cac tgc tac gct ccc aac gtc gac tgg acc gtg ctc 1016  
 Ser Val Ala Trp His Cys Tyr Ala Pro Asn Val Asp Trp Thr Val Leu  
 285 290 295

agc cag ttc cac aac aca aac cct gga gtg aag caa tat atg acc gag 1064  
 Ser Gln Phe His Asn Thr Asn Pro Gly Val Lys Gln Tyr Met Thr Glu  
 300 305 310 315

tgc tgg act cca gca tct ggc gca tgg cat cag gcg gcg gac ttc acc 1112  
 Cys Trp Thr Pro Ala Ser Gly Ala Trp His Gln Ala Ala Asp Phe Thr  
 320 325 330

atg ggt ccc ctg cag aac tgg gcc tcg gga gtg gca gca tgg act ctg 1160  
 Met Gly Pro Leu Gln Asn Trp Ala Ser Gly Val Ala Ala Trp Thr Leu  
 335 340 345

gga acc aac gct cag gat ggt ccg cat ctg tcc act ggc ggc tgc gcg 1208  
 Gly Thr Asn Ala Gln Asp Gly Pro His Leu Ser Thr Gly Gly Cys Ala  
 350 355 360

aca tgt caa ggc ttg gtg acc atc aac aac gga gga tac acg ctc aac 1256  
 Thr Cys Gln Gly Leu Val Thr Ile Asn Asn Gly Gly Tyr Thr Leu Asn  
 365 370 375

acc gca tac tac atg atg gcg caa ttc agc aag ttc atg ccg cct ggt 1304  
 Thr Ala Tyr Tyr Met Met Ala Gln Phe Ser Lys Phe Met Pro Pro Gly  
 380 385 390 395

gcg att gtg ctc aat ggc agt ggc agc tac aag tac tct ggc gga ggc 1352  
 Ala Ile Val Leu Asn Gly Ser Gly Ser Tyr Thr Tyr Ser Gly Gly Gly  
 400 405 410

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ggt atc cag tcc gtg gct tcc ttg aat occ gat gga acc cgc act gtg 1400  
 Gly Ile Gln Ser Val Ala Ser Leu Asn Pro Asp Gly Thr Arg Thr Val  
 415 420 425  
 gtt att gaa aac act ttt ggc aat gat gtc tat gtg act gtc act atg 1448  
 Val Ile Glu Asn Thr Phe Gly Asn Asp Val Tyr Val Thr Val Thr Met  
 430 435 440  
 aag agc ggg cag aag tgg agt ggg aac gcc cct agc caa tcc gtg act 1496  
 Lys Ser Gly Gln Lys Trp Ser Gly Asn Ala Pro Ser Gln Ser Val Thr  
 445 450 455  
 acc tgg gtt ctt cca tct gct tga aaagagtga gtttcagatg gttagatatg 1550  
 Thr Trp Val Leu Pro Ser Ala  
 460 465  
 tattgaagag tagcgcttgg agacatcaat agcctttttc taattacatg togtgcagct 1610  
 tccaaaaaaa aaaaaaaaaa aaaaaaaaaa aactcga 1647

SEQ ID NO: 10

Met Arg Ile Ser Val Gly Ala Leu Leu Gly Leu Thr Ala Leu Ser His  
 1 5 10 15  
 Ala Thr Thr Glu Lys Arg Ala Ala Ser Ala Ser Ala Tyr Cys Ser Asn  
 20 25 30  
 Ser Ala Gly Asn Tyr Lys Leu Ser Ser Ile Ala Ala Pro Val Gln Gly  
 35 40 45  
 Ala Gly Asn Pro Gly Ser Glu Ser Thr Trp Gln Leu Thr Val Asp Asp  
 50 55 60  
 Thr Ser Ser Gly His Lys Gln Thr Ile Val Gly Phe Gly Ala Ala Val  
 65 70 75 80  
 Thr Asp Ala Thr Val Thr Ser Phe Asn Thr Leu Ser Ala Ser Val Leu  
 85 90 95  
 Gln Asp Leu Leu Asn Lys Leu Met Thr Pro Ala Gly Ala Asn Phe Ala  
 100 105 110  
 Leu Met Arg His Thr Ile Gly Ala Ser Asp Leu Ser Gly Asp Pro Ala  
 115 120 125  
 Tyr Thr Tyr Asp Asp Asn Gly Gly Lys Ala Asp Pro Ser Leu Ser Gly  
 130 135 140  
 Phe Asn Leu Gly Asp Arg Gly Thr Ala Met Ala Lys Met Leu Ala Thr  
 145 150 155 160  
 Met Lys Ser Leu Gln Pro Asn Leu Lys Ile Leu Gly Ser Pro Trp Ser  
 165 170 175  
 Ala Pro Gly Trp Met Lys Leu Asn Gly Val Leu Asp Gly Asn Thr Asn  
 180 185 190

Asn Asn Asn Leu Asn Asp Gly Tyr Leu Thr Ser Gly Gly Thr Gly Ser  
 195 200 205  
 Thr Gly Tyr Ala Ser Gln Phe Ala Gln Tyr Phe Val Lys Tyr Ile Gln  
 210 215 220  
 Ala Tyr Lys Asn Leu Gly Ala His Val Asp Ala Ile Thr Ile Gln Asn  
 225 230 235 240  
 Glu Pro Leu Phe Ser Ser Ala Gly Tyr Pro Thr Met Tyr Val Tyr Asp  
 245 250 255  
 Tyr Glu Ser Ala Gln Leu Ile Gln Asn Tyr Ile Gly Pro Ala Leu Ala  
 260 265 270  
 Ser Ala Gly Leu Asp Thr Glu Ile Trp Ala Tyr Asp His Asn Thr Asp  
 275 280 285  
 Val Pro Ser Tyr Pro Gln Thr Val Leu Asn Gln Ala Gly Gln Tyr Val  
 290 295 300  
 Lys Ser Val Ala Trp His Cys Tyr Ala Pro Asn Val Asp Trp Thr Val  
 305 310 315 320  
 Leu Ser Gln Phe His Asn Thr Asn Pro Gly Val Lys Gln Tyr Met Thr  
 325 330 335  
 Glu Cys Trp Thr Pro Ala Ser Gly Ala Trp His Gln Ala Ala Asp Phe  
 340 345 350  
 Thr Met Gly Pro Leu Gln Asn Trp Ala Ser Gly Val Ala Ala Trp Thr  
 355 360 365  
 Leu Gly Thr Asn Ala Gln Asp Gly Pro His Leu Ser Thr Gly Gly Cys  
 370 375 380  
 Ala Thr Cys Gln Gly Leu Val Thr Ile Asn Asn Gly Gly Tyr Thr Leu  
 385 390 395 400  
 Asn Thr Ala Tyr Tyr Met Met Ala Gln Phe Ser Lys Phe Met Pro Pro  
 405 410 415  
 Gly Ala Ile Val Leu Asn Gly Ser Gly Ser Tyr Thr Tyr Ser Gly Gly  
 420 425 430  
 Gly Gly Ile Gln Ser Val Ala Ser Leu Asn Pro Asp Gly Thr Arg Thr  
 435 440 445  
 Val Val Ile Glu Asn Thr Phe Gly Asn Asp Val Tyr Val Thr Val Thr  
 450 455 460  
 Met Lys Ser Gly Gln Lys Trp Ser Gly Asn Ala Pro Ser Gln Ser Val  
 465 470 475 480  
 Thr Thr Trp Val Leu Pro Ser Ala  
 485

### Example 9

#### Confirmation of the expression of $\beta$ -primeverosidase in mold

##### a) Construction of expression cassette

In order to verify whether or not the cloned gene is the primeverosidase gene, expression of the thus obtained DNA was confirmed. Using an *Aspergillus oryzae* Taka-amylase gene-containing plasmid pTG-Taa (Kato M, Aoyama A, Naruse F, Kobayashi T and Tsukagoshi N (1997), An *Aspergillus nidulans* nuclear protein, An CP, involved in enhancement of Taka-amylase A gene expression binds to the CCAAT-containing *taaG2*, *amdS* and *gata* promoters., *Mol. Gen. Genet.*, 254: 119 - 126) as the template, a fragment was obtained by amplifying it by PCR using

a primer TAA5'

SEQ ID NO: 10 //

sense primer:

5'-GGGCCTGCAGGAATTCATGGTGTT-3'

and a primer TP3'

SEQ ID NO: 11/12

antisense primer:

5'-CGAGCCGGGGTTTCCGTCCGCAGGCGTTGC-3'.

<PCR reaction solution>

template DNA solution (50 $\mu$ g/ml)	1 $\mu$ l
50 $\mu$ M sense primer	1 $\mu$ l
50 $\mu$ M antisense primer	1 $\mu$ l

sterilized water 22 µl  
Premix Taq (EX Taq Version TaKaRa) 23 µl

<PCR reaction conditions>

Stage 1: denaturation (95°C, 1 minute) 1 cycle  
Stage 2: denaturation (95°C, 1 minute) 30 cycles  
annealing (55°C, 1 minute)  
elongation (72°C, 1 minute)

Stage 3: elongation (72°C, 5 minutes) 1 cycle

Also, a fragment was obtained by amplifying it by PCR using the DNA-containing plasmid pAFPri as the template and using a primer dPC5'

SEQ ID NO: 12/13 ✓

sense primer:

5'-GCAACGCCTGCGGACGGAACCCCGGCTCG-3'

and a primer PC3' ✓

SEQ ID NO: 13/14

antisense primer:

5'-GCGCAAGCTTGGGAAGCTGCACGACATGTAA-3'.

In addition, after recovering and mixing respective fragments, a fragment was obtained by amplifying it by PCR using the primer TAA5' and primer PC3'. This fragment contains a sequence corresponding to a region of from the *Aspergillus oryzae* Taka-amylase promoter to the N-terminal 5th amino acid of the mature protein and a sequence corresponding to a region of from the N-terminal 28th amino



acid (glycine) to the C-terminal of the mature  $\beta$ -primeverosidase protein. An *Sse8387I* site has been introduced into the upstream of the thus obtained fragment, and a *HindIII* site into its downstream. The fragment was recovered by treating with restriction enzymes *Sse8387I* and *HindIII*.

Regarding the terminator region, a fragment was obtained by amplifying it by PCR using pTG-Taa as the template and using a primer TAAH

SEQ ID NO: 14 15

sense primer:

5'-GCGCAAGCTTTGAAGGGTGGAGAGT-3'

and a primer TAA3'

SEQ ID NO: 15 16

antisense primer:

5'-GCGCCCTGCAGGTCTAGAATTCCTAGTGGTT-3'.

A *HindIII* site has been introduced into the upstream of the thus obtained fragment, and an *Sse8387I* site into its downstream. The fragment was recovered by treating with restriction enzymes *HindIII* and *Sse8387I*.

A plasmid pTG1 containing orotidine-5'-phosphate decarboxylase gene (pyr4) as a marker gene (Kato M (1997), *Mol. Gen. Genet.*, 254: 119 - 126) was treated with the restriction enzyme *Sse8387I* and with alkaline phosphatase and then recovered.

Plasmids pAFPriE1 (forward direction to the direction of the marker gene) and pAFPriE2 (reverse direction) were obtained by connecting these 3 fragments.

b) Acquisition of transformant

An orotidine-5'-phosphate decarboxylase (pyrG) producing strain *Aspergillus nidulans* G191 (Kato M (1997), *Mol. Gen. Genet.*, 254: 119 - 126) was inoculated into a complete medium (2% malt extract, 0.1% peptone, 2% glucose, 0.1% uridine, 2 µg/ml p-aminobenzoic acid, pH 6.5) and cultured at 30°C for 18 hours on a shaker. The cells were collected by filtration, suspended in a protoplast solution (0.8 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM CaCl<sub>2</sub>, 3.75 mg/ml Novozyme 234) and then treated at 30°C for 1 hour on a shaker. The resulting protoplasts were recovered by filtration and centrifuged at 1,500 rpm for 5 minutes to obtain the protoplasts as the precipitate. This precipitate was suspended in 0.8 M NaCl solution and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. This was again suspended in 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution and centrifuged at 1,500 rpm for 5 minutes to collect the precipitate. A protoplast solution was obtained by suspending this in an appropriate amount of 0.8 M NaCl/50 mM CaCl<sub>2</sub> solution. Next, 50 µl of this protoplast solution was mixed with 20 µg of a DNA solution and 12.5 µl of a PEG solution (25% PEG 6000/50 mM CaCl<sub>2</sub>/10 mM Tris-HCl (pH 7.5))



shaker. The culture broths were sampled after 48, 72, and 96 hours and filtered, and the resulting filtrates were checked for the activity. The enzyme activity was not found in the transformants obtained from pTG1 and pAFPriE2 but the enzyme activity was confirmed in 5 transformant strains obtained from pAFPriE1.

#### Example 10

##### Comparison with plant gene by a hybridization method

Using the gene of an enzyme similar to the tea-derived diglycosidase as the probe, an examination was carried out to know if a gene having a similar structure is present on the chromosome of the microorganisms in which the presence of diglycosidase had been confirmed by us. Preparation of gene fragment of an enzyme similar to the tea-derived diglycosidase was carried out with reference to the report by Sakata, Mizutani *et al.* (The 73rd Annual Meeting of Agricultural Chemical Society of Japan) and Japanese Patent Application No. Hei. 11-56299.

Preparation of microorganism-derived chromosome was carried out in the following manner.

Preparation of chromosomes from yeast and fungi was carried out in accordance with the method described in *Molecular and Cellular Biology*, Vol. 3, pp. 1430 - 1439 (1983). Preparation of chromosomal DNA from bacteria was carried out in accordance with the method of Saito and

Mitsuura (Biochim. Biophys. Acta, Vol. 72, pp. 619 - 629, 1963). Preparation of chromosomal DNA from actinomycetes was carried out in accordance with the method of Iefuji et al. (Biosci. Biotec. Biochem., Vol. 60, pp. 1331 - 1338, 1996).

A 10 µg portion of each of the thus obtained various chromosomal DNA preparations was digested with BamHI in the case of *Aspergillus fumigatus*, *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus aculaetus*, *Penicillium lilacinum*, *Penicillium decumbence*, *Penicillium multicolor*, *Talaromyces emersonii*, *Mortierella vinacea*, *Cryptococcus albidus*, *Corynebacterium ammoniagenes*, *Corynebacterium glutamicum*, *Microbacterium arborescens* and *Penicillium rugulosum*, or with EcoRI in the case of *Rhizopus oryzae*, *Rhizomucor pusillus*, *Rhizomucor miehei* and *Actinoplanes missouriensis*, and the resulting digest was applied to a 1% agarose gel electrophoresis. As a control, the gene fragment of an enzyme similar to the tea-derived diglycosidase used as the probe was also subjected to the same gel electrophoresis. After the electrophoresis, DNA samples were blotted on a nylon membrane and hybridization was carried out using a labeled gene fragment p of an enzyme similar to the tea-derived diglycosidase (structural gene moiety of matured plant primeverosidase gene) as the probe, using DIG System Kit (Boehringer Mannheim) in

accordance with the instruction attached thereto. As a result, when the detection was carried out under hybridization conditions (5 x SSC, 1% blocking agent, 0.1% N-lauroylsarcosine sodium, 0.02% SDS, 68°C, overnight) and washing conditions (6 x SSC, 0.1% SDS, room temperature, 5 min. x 2 and 6 x SSC, 0.1% SDS, 45°C, 15 min. x 2), a signal was obtained at a position where the plant gene was blotted, but the signal was not observed at any other position where the microorganism-derived genome was blotted. Thus, it is considered that the microorganism-derived diglycosidase gene has a structure which is different from the plant primeverosidase gene.

On the other hand, using the *Aspergillus fumigatus* IAM 2020 diglycosidase gene of the invention obtained in Example 8 as the probe, an examination was carried out by the same methods and conditions to know if a gene having a similar structure is present on the chromosome of the microorganisms in which the presence of diglycosidase had been confirmed by us. As a result, the signal was detected in these microorganisms.

In addition, it was able to detect the signal in *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus aculeatus*, *Penicillium multicolor*, *Penicillium lilacinum*, *Corynebacterium ammoniagenes* and *Corynebacterium glutamicum*, even under more stringent washing conditions (5

x SSC, room temperature, 10 min. and 4 x SSC, 68°C, 30 min.).

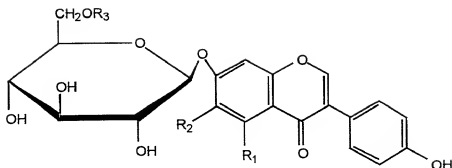
Example 11

Activity of the diglycosidase to hydrolyze isoflavone in isoflavone glycosides

As shown in the following table, glucosides and modified glucosides of acetylglucosides and malonylglucosides, are present in isoflavone glycosides. The activity of diglycosidase to hydrolyze the acetyl type and malonyl type glucosides, namely the aglycon releasing activity, was examined.

Isoflavone	M. wt.	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Concentration
Glycitin	446.4	H	OCH <sub>3</sub>	H	2 mM
Genistin	432.4	OH	H	H	2 mM
Daidzin	416.4	H	H	H	2 mM
Acetylglycitin	458.4	H	OCH <sub>3</sub>	COCH <sub>3</sub>	2 mM
Acetylgenistin	474.7	OH	H	COCH <sub>3</sub>	2 mM
Acetyl daidzin	458.4	H	H	COCH <sub>3</sub>	2 mM
Malonylglycitin	502.4	H	OCH <sub>3</sub>	COCH <sub>2</sub> COOH	2 mM
Malonylgenistin	518.4	OH	H	COCH <sub>2</sub> COOH	2 mM
Malonyl daidzin	502.4	H	H	COCH <sub>2</sub> COOH	2 mM

R<sub>1</sub> to R<sub>3</sub> correspond to the following structural formula.



Each of acetylglycitin, acetylgenistin, acetyl daidzin, malonylglycitin, malonylgenistin and malonyl daidzin (produced by Fujicco, available from Nakalai Tesque) was allowed to react with a diglycosidase enzyme solution prepared from *Asp. fumigatus* or *Pen. multicolar* or with an almond-derived glucosidase (mfd. by Sigma) under the following conditions.

Each of the enzymes was diluted with 20 mM acetate buffer (pH 4.0) to adjust its activity to 1.88 AU/ml, and then 2 mM of each isoflavone (12.5  $\mu$ l), 20 mM of acetate buffer (87.5  $\mu$ l) and each enzyme solution (25  $\mu$ l) were mixed to carry out the reaction at 55°C. After 1, 3 and 6 hours of the reaction, samples were taken out in 25  $\mu$ l portions, and each of the samples was mixed with 75  $\mu$ l of methanol and 900  $\mu$ l of water, filtered through a filter (0.2  $\mu$ m) and then further diluted 2.5 times with water. A 1 ml portion thereof was analyzed by HPLC (HPLC conditions; column: ODS 80TM (Tosoh), eluent: a mixed solution of



acetonitrile and 10% acetic acid, separation under linear density gradient).

As a result, it was revealed that the glucosidase (Sigma) hardly hydrolyzed the modified glucoside substrates, but both diglycosidase enzymes hydrolyzed all of the modified glucosides efficiently and released the aglycon.

#### Example 12

##### Preparation of an aroma component precursor, eugenyl primeveroside

A 2 kg portion of fresh leaves of *Camellia sasanqua* were extracted with hot water at 100°C for 10 minutes, and the extract was applied to a column packed with Diaion HP20 (mfd. by Mitsubishi Chemical) to adsorb eugenyl primeveroside thereon. The column was washed with about 2 times the bed volume of deionized water and 20% methanol and then the adsorbed eugenyl primeveroside was eluted with 100% methanol. Thereafter, the thus recovered methanol solution containing eugenyl primeveroside was concentrated to crystallize eugenyl primeveroside which was then recovered using a glass filter.

A 1 ml portion of the crude enzyme concentrate obtained in Example 4 was mixed with 1 ml of the eugenyl primeveroside solution adjusted to 5 mg/ml with 20 mM phosphate buffer (pH 6.0), the mixture was incubated at

37°C for 24 hours and then the formation of aroma was examined by a sensory test (10 panel). As a result, formation of the eugenol-specific aroma was confirmed in all of the cases in which the crude enzyme concentrates of two *Aspergillus niger* strains and *Aspergillus fumigatus* were used.

In a case in which the crude enzyme concentrates were used after their heat-treatment (100°C, 10 minutes), the eugenol-specific aroma was not observed. Accordingly, it was found that these crude enzyme extracts have a function to release the aroma component aglycon from glycosides such as eugenyl primeveroside.

#### Example 13

#### Release of disaccharide from pNP-primeveroside by purified enzyme

A 0.3 AU portion of the purified enzyme solution obtained in Example 6 and the aforementioned pNP-primeveroside were incubated at 37°C for 24 hours to examine release of disaccharide by TLC. As a result, a spot was observed at the same position of primeverose so that release of a disaccharide was confirmed. Such a spot was not found by the heat-treated purified enzyme solution used as a control. Thus, it was revealed that the purified enzyme has a function to release a disaccharide from a disaccharide glycoside.

Example 14

Release of disaccharide and formation of aroma from eugenyl  
primeveroside by purified enzyme

A 0.3 AU portion of the purified enzyme solution obtained in Example 6 and the aforementioned PNP-primeveroside were incubated at 37°C for 24 hours to examine release of disaccharide by TLC. As a result, a spot was observed at the same position of primeverose so that release of a disaccharide was confirmed. In addition, when the reaction solution was analyzed by a gas chromatography, release of eugenol as the aglycon of eugenyl primeveroside glycoside was confirmed, and the release of eugenol was also confirmed by a sensory test. These were not found in heat-inactivated enzyme solution. Thus, it was revealed that the purified enzyme forms aroma by acting upon an aroma precursor such as eugenyl primeveroside.

Example 15

Release of disaccharide from pigment glycoside

Release of disaccharide was examined using a disaccharide glycoside, ruberythric acid, as the substrate. Ruberythric acid was prepared by adsorbing a water extract of *Rubia tinctorum* L. root powder for staining use (available from Tanaka Senshoku Ten) to HP-20 column, washing the column with 50% methanol, and then eluting the

compound with 100% methanol and evaporating the eluate to dryness using an evaporator. A substrate prepared by dissolving the thus recovered ruberythric acid in a phosphate buffer to a concentration of 5 mg/ml was mixed with the crude enzyme solution (0.3 AU) shown in Example 4 or the purified enzyme solution shown in Example 6 and incubated at 37°C for 24 hours, and then the reaction solution was analyzed by TLC. As a result, release of the disaccharide primeverose and the aglycon alizarin was observed by the crude enzyme solution and purified enzyme solution.

#### Example 16

##### Hydrolysis of other disaccharide glycosides

Using the diglycosidase preparations derived from various microorganisms shown in Example 5, their ability to hydrolyze various disaccharide glycosides was examined using TLC. As a result, it was revealed that the diglycosidase acts upon not only the primeveroside glycosides but also various other disaccharide glycosides analogous to the primeveroside glycosides, including rutinose glycosides such as naringin and rutin, gentiobiose glycosides, arabinofuranosyl glycosides and aviofuranosyl glycosides, and thereby releases disaccharides and produces respective free aglycons.

Example 17

Improvement of tea extract aroma

Using the *Aspergillus fumigatus* enzyme solutions shown in Examples 4 and 6, their function to increase aroma components of green tea, black tea and oolong tea was examined. Each tea extract was mixed with 1.88 AU of the enzyme and incubated at 55°C for 24 hours and then increase in the aroma was examined by a gas chromatography under the aforementioned conditions. As a result, it was found that aroma components such as 1-hexanol, 3-hexen-1-ol, benzaldehyde, linalool, methyl salicylate, geraniol and benzyl alcohol were increased. Increase in the aroma was also found by a sensory test.

Example 18

Improvement of fruit juice aroma

Using the *Aspergillus fumigatus* and *Penicillium multicolor* enzyme solutions shown in Examples 4 and 5, 1.88 AU of each of the enzyme was added to a fruit juice such as of grape, orange, apple, prune or nectar and incubated at 37°C for 24 hours and then the aroma was analyzed by a gas chromatography. As a result, increase in the aroma components such as linalool was observed. Improvement of the aroma was also found in the enzyme-treated fruit juices by a sensory test.

Example 19

Improvement of wine aroma

Using the various crude enzyme solutions shown in Example 4, 0.5 AU of each enzyme was added to red wine and white wine and incubated at 37°C for 24 hours to examine improvement of the aroma by a sensory test. As a result, improvement of the aroma was found in both cases.

Example 20

When 1 ml of each of the various crude enzyme concentrates obtained in Examples 4 and 5 was mixed with 1 ml of a grape juice (commercial product: 100% fruit juice, concentrated and reduced) and then incubated at 37°C overnight (14 hours) to examine the aroma, the aroma was clearly improved in comparison with a sample in which an acetate buffer was added instead of the enzyme preparation. In addition, this function was not found when the crude enzyme concentrate was heat-treated at 100°C for 10 minutes.

Example 21

A 1 ml portion of each of the crude enzyme concentrates obtained in Example 4 was mixed with 1 ml of a commercially available orange juice (reduced concentrate) and incubated at 37°C for 24 hours to examine formation of the aroma by a sensory test. As a result, improving effect of the aroma of the orange juice was found in the crude



acid:methanol = 1:4 solution was sprayed thereto after the development and allowed to stand at 105°C for 10 minutes).

As a result, it was revealed that  $\beta$ -primeverosidase transfers the diglycoside to phenethyl alcohol and thereby forms a disaccharide glycoside.

#### Industrial Applicability

An enzyme having a function to cut  $\beta$ -primeveroside and/or analogous disaccharide glycoside in disaccharide unit or to hydrolyze modified glucosides can be provided by the invention as a novel enzyme using microorganisms as its supply source, and it can be broadly used in various types of food, medicaments, quasi drugs and the like by using the enzyme composition of the invention. For example, the aroma, pigment and physiologically active component of food can be increased or decreased.

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2. The enzyme according to claim 1, wherein said disaccharide glycoside is  $\beta$ -primeveroside and/or an analogous disaccharide glycoside.

4. A polypeptide which comprises a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing.

6. A polynucleotide which comprises a polynucleotide being selected from the following

polynucleotides (a) to (g) and encoding a polypeptide having an activity to act upon a disaccharide glycoside to release saccharides from said disaccharide glycoside in disaccharide unit;

(a) a polynucleotide which encodes a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing,

(b) a polynucleotide which encodes a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing, wherein one or more amino acid residues of the amino acid sequence are modified by at least one of deletion, addition, insertion and substitution,

(c) a polynucleotide which has the nucleotide sequence of SEQ ID NO: 7 shown in the Sequence Listing,

(d) a polynucleotide which has the nucleotide sequence of SEQ ID NO: 7 shown in the Sequence Listing, wherein one or more bases of the nucleotide sequence are modified by at least one of deletion, addition, insertion and substitution,

(e) a gene which hybridizes with any one of the aforementioned polynucleotides (a) to (d) under a stringent condition,

(f) a polynucleotide which has homology with any one of the aforementioned polynucleotides (a) to (d), and

(g) a polynucleotide which is degenerate with respect to any one of the aforementioned polynucleotides (a) to (f).

7. A polynucleotide which comprises a polynucleotide coding for a polypeptide having the amino acid sequence of SEQ ID NO: 8 shown in the Sequence Listing.

8. A recombinant vector which contains the polynucleotide of any one of claims 5 to 7.

9. A transformant transformed with the recombinant vector of claim 8.

10. A method for producing an enzyme having an activity to act upon a disaccharide glycoside to release saccharides from said disaccharide glycoside in disaccharide unit, which comprises culturing the transformant of claim 9 to effect production of the enzyme having an activity to act upon a disaccharide glycoside to release saccharides from said disaccharide glycoside in disaccharide unit, and subsequently collecting said enzyme from the resulting culture mixture.

11. A method for producing an enzyme having an activity to act upon a disaccharide glycoside to release saccharides from said disaccharide glycoside in disaccharide unit, which comprises culturing a microorganism in a nutrient medium to effect production of the enzyme having an activity to act upon a disaccharide

glycoside to release saccharides from said disaccharide glycoside in disaccharide unit, and subsequently collecting said enzyme from the resulting culture mixture.

12. The production method according to claim 11, wherein the microorganism is selected from the genus *Aspergillus*, the genus *Penicillium*, the genus *Rhizopus*, the genus *Rhizomucor*, the genus *Talaromyces*, the genus *Mortierella*, the genus *Cryptococcus*, the genus *Microbacterium*, the genus *Corynebacterium* and the genus *Actinoplanes*.

13. The method for producing an enzyme having an activity to act upon a disaccharide glycoside to release saccharides from said disaccharide glycoside in disaccharide unit according to claim 10, 11 or 12, wherein the nutrient medium contains a substance which induces production of an enzyme having an action to release saccharides from a disaccharide glycoside in disaccharide unit.

14. The method for producing a novel enzyme composition according to claim 13, wherein the inducer is a saccharide.

15. A method for modifying composition of a material containing a disaccharide glycoside, which comprises allowing an enzyme having an action to release

saccharides from said disaccharide glycoside in  
disaccharide unit to react with said material.

16. The modifying method according to claim 15, wherein said disaccharide glycoside is an aromatic component precursor or a pigment component precursor.

17. The modifying method according to claim 15, wherein said disaccharide glycoside is  $\beta$ -primeveroside and/or an analogous disaccharide glycoside.

18. A method for modifying composition of a material containing a modified monoglucoside, which comprises allowing an enzyme having an action to release a modified monosaccharide unit from said modified monoglucoside to react with said material.

19. The method of claim 18, wherein said modified monoglucoside is at least one member selected from the group consisting of acetylglucoside, malonylglucoside, methylglucoside, phosphoglucoside, and amidoglucoside.

20. The method of claim 19, wherein said modified monoglucoside is acetylglucoside of isoflavone, malonylglucoside of isoflavone, and both thereof.

# ABSTRACT

This invention relates to a novel microorganism-derived enzyme having an activity to cut disaccharide glycosides (particularly,  $\beta$ -primeveroside and/or analogous disaccharide glycosides) in disaccharide unit, a method for producing the enzyme, a gene which encodes the enzyme and use of the enzyme. Various components can be formed by the action of this enzyme upon disaccharide glycosides.

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# DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NOVEL ENZYME COMPOSITION AND PRODUCTION METHOD

AND USE THEREOF

the specification of which is attached hereto unless the following box is checked:

☒ was filed on September 29, 1999 as United States Application Number or PCT International Application Number PCT/JP99/05346 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which is material to the patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

P. Hei. 10-294675

Japan

30/September/1998

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

☒ hereby claim the benefits under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)

I hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexico, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,773; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,523; Waddell A. Biggart, Reg. No. 24,851; Louis Gubinsky, Reg. No. 24,833; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 25,703; John R. Inge, Reg. No. 26,918; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,186; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765 and Robert M. Masters, Reg. No. 35,603; my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to SUHRUE, MION, ZINN, MACPEAK & SEAS, PLLC, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date March 19, 2001

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First Name Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

Date \_\_\_\_\_ Eighth Inventor \_\_\_\_\_  
First Name Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

(Supply similar information for ninth and subsequent joint inventors.)



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